The Proteolytic and Saccharolytic Activity of some Natural Waters and their Associated Bacteria

by:

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DECLARATION

In accordance with the regulations for the degree of Doctor of Philosophy of the University of Warwick, I declare that full reference has been made throughout this thesis to all published and unpublished sources used and that all advice and assistance has been acknowledged. Otherwise the results presented are based on my own research carried out in the Malvern and Tewkesbury laboratories of the Severn-Trent Water Authority from January 1981 to December 1985. Certain information was obtained using autoanalytical equipment operated by the Severn-Trent Water Authority at the Malvern Laboratory but all sample preparation and processing of data output from this equipment was carried out by myself. This thesis has not been submitted previously for a Degree of this or any other University.

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SUMMARY

This study is concerned with the relationship between two hydrolytic enzyme activities, saccharase and protease and other limnological factors, including bacterial flora, in natural waters of varying degrees of organic pollution.

Published methodologies for measuring proteolytic and saccharolytic activities were employed to yield data whose statistical distribution was found to be modelled by a log-normal distribution.

Natural activities were correlated with biochemical oxygen demand, suspended solids, viable bacteria, combined nitrogen, dissolved solids, flow and temperature.

Regression equations are formulated with biochemical oxygen demand and bacterial numbers either singly or combined explaining most of the variation in enzyme activity. Other contributory factors were suspended solids, flow and temperature. These equations could predict enzyme activity at other sites with acceptable levels of confidence.

The best predictive equations for enzyme activities were:

$$\log \text{Saccharase} = 0.2 \log \text{BOD} + 0.1 \log \text{Suspended solids} + 0.8 \log \text{Viable count} + 0.9$$

$$\text{Protease} = -469 \text{ Temperature} + 56708 \text{ Flow} + 443 \text{ BOD} - 1.54 \text{ Viable count} + 6853$$

Aquatic bacteria producing enzyme activity were isolated from watercourses and identified as belonging to the genera Pseudomonas, Flavobacterium and Flexibacter. When grown in defined media, enzyme production was stimulated by the addition of sucrose or casein to the medium. In the case of one organism casein could act as sole source of carbon and nitrogen.

Extracts of cell suspension were shown to have enzyme activities. When investigated these activities had maximum values dependent on pH and temperature. This pH and temperature profile of activity was also demonstrated in samples of river water. Enzyme extracts followed Michaelis-Menten reaction kinetics with Michaelis' constants similar to those reported in comparable extracts.

ABBREVIATIONS

Abbreviations used in this thesis are listed as follows:

ATP	adenosine triphosphate
BOD	biochemical oxygen demand in 5 days at 20°C
BOD(ATU)	biochemical oxygen demand with nitrification suppressed by the addition of allylthiourea
CFU	colony forming units
DNA	deoxyribonucleic acid
EColi	Escherichia coli
ETS	electron transport system
OD	optical density
RNA	ribonucleic acid
TCA	trichloroacetic acid
TON	total oxidised nitrogen
μm	10^{-6} metre
nm	10^{-9} metre
°C	degrees Celsius

1 INTRODUCTION

1.1 POLLUTION CONTROL CONTEXT

1.1.1 General Introduction

Lakes ,rivers and oceans have been intensively studied over a long period because of their economic and political importance and intrinsic scientific interest. Wetzel (1975) defined rivers as unidirectional running water systems, flowing in narrow parallel sided troughs and despite only constituting 0.0001% of the Earth's water they pass thirty times their contained volume each year.

Rivers are multi-use systems with certain uses being mutually antagonistic (Oglesby et_al 1972). The use of rivers as drainage systems for clean and dirty water can conflict with their use for abstraction for potable water supply or crop irrigation. The impact on river features such as channel character, water quantity and regime, water quality, biota and aesthetic appeal, of various human manipulations tends to have a nett harmful effect. There is often a disruption of a naturally stable but dynamic equilibrium. Attempts are often made to simplify a complex natural situation resulting in a stimulation of reaction towards the original state. For example the creation of smooth trapezoidal channels by clearance of vegetation can stimulate excessive weed growth and consequent silting problems. (Robinson 1986) Multiple use of rivers has stimulated study into the physical, chemical and biological nature of riverine systems.

Physical studies employ geomorphological methods to describe channel shape pattern and evolution. Hydraulic properties of rivers are important in relation to land drainage, flood control, power generation and water resources. Stream flow measurement, including modelling of rainfall and catchment run-off, is a well established discipline providing a hydrological perspective on rivers.

Chemical investigation of rivers would be concerned with the nature and concentration of particulate and soluble matter, both organic and inorganic. The background chemical profile would reflect lithology and land use of the catchment and the character of the atmospheric input. In acquiring this information account will have to be taken of the inherent dynamic nature of most flowing water systems.

Aquatic habitats are created by the combination of physical and chemical dimensions. As both of these dimensions have a dynamic character the resultant flora and fauna will tend to be very varied both qualititatively and quantitatively.

Historically the search to know more about the biology of rivers, lakes and the sea has been driven by a need to understand the sanitary aspects. An early text such as Whipple (1927), first published in 1899, has a wide ranging description of organisms such as algae, protozoa and bacteria important to the waterworks chemist, biologist and engineer. Also included are the physical and chemical limnology of rivers, lakes and reservoirs and self purification of streams. It is a reflection of the depth of current knowledge that no single text today could cover such a wide spread of aquatic ecological subject matter.

Pioneering investigation of the natural history of water bourne disease, water pollution and water purification systems resulted in the early counting and identification of aquatic micro-organisms (Koch 1884; Frankland and Frankland 1894). In this country the sanitary aspects of water bacteriology were summarised in 1934 in the Ministry of Health's report on The Bacteriological Examination of Water Supplies.

Research which started in these areas branched out to consider the more general ecology of aquatic systems. Aspects covered included primary productivity in relation to eutrophication in lakes such as Zurichsee (Hasler 1947); input of allochthonous organic matter from natural sources or anthropogenic sources such as sewage with subsequent heterotrophic breakdown causing graded changes in stream animal and plant communities (Kolkwitz and Marsson 1902).

Detritus food chains were discovered to be of crucial importance in cycling of organic and inorganic nutrients (Zobell and Feltham 1942).

Methods of study have tended to be analytical but in breaking down the components, information is lost as to relationships both functional and structural. In contrast the so called Holistic or systems approaches (Heath 1979) should be more successful in describing aquatic units but are faced with much inherent variability and the need for large amounts of raw data to separate true observation from environmental noise.

The degree of success of the above approaches when applied to the solution of practical problems can be judged by looking at the effective exploitation of water resources for public water supply on the one hand and the continuing and in some cases worsening problems of eutrophication or gross pollution of rivers and oceans on the other.

1.1.2 Bacteria Associated With Natural Waters

Following the pioneering work of the bacteriologists in the mid nineteenth Century on human disease, attention was focussed on problems of water-borne diseases. New techniques were used to study bacteria present in water then decisions could be made whether water was bacteriologically safe for human consumption. At this time there was little work on the growth of bacteria in natural waters, their biochemical activities or relationships with other organisms living in the waters.

Collins (1963) reviewed the early work on the ecology of freshwater bacteria and noted that much of the work in the first part of this century was carried out in the United States and Europe; concentrating on bacteria in lakes. The work of Fred et al (1924) on Lake Mendota in Wisconsin showed that lake water in culture could actually support greater populations of bacteria than are found in situ. Bacterial numbers were found to increase in spring and autumn largely due to soil bacteria being washed into lakes and subsequently multiplying. In contrast Henrici (1938) made counts of bacteria at varying water depths at a time when there was no landward input of bacteria as streams were dry. It was found that bacterial numbers followed general plankton

counts, with the conclusion that plankton algae probably produced organic matter that could be used by lake bacteria.

Early studies tended to concentrate on pathogenic organisms and the cultural conditions employed would not select for all indigenous organisms. Frost and Streeter (1924) investigated die off or disinfection of bacteria in the Ohio River in the United States. They were concerned to distinguish between bacteria of sewage as opposed to a more natural river origin. Growth at 20° on a gelatin medium was used to count indigenous bacteria, growth at 37° on nutrient agar to count those organisms which could be pathogenic and a standard test of E Coli. the classic indicator of sewage pollution. Subsequent work diversified to include study of all types of bacteria found in natural waters whether of indigenous origin or as inputs from other sources.

More recent work has attempted to describe the microbial flora of rivers and lakes both qualitatively and quantitatively. progress has been made in both areas despite a warning (Wuhrman 1964), that the majority of bacteria in rivers are washed in from soil rendering identification of little value because their role in the river environment would not necessarily reflect their role in soils. A complementary warning was issued by Jannasch (1969) that there were doubts about the validity of counting bacteria because no information was thus provided on in situ microbial activity.

Identification of bacterial genera and species found in natural waters starts by searching for the optimum cultural conditions for colonial outgrowth of recovered organisms. There are many variations on the theme of media composition, incubation, time

and temperature. Under aerobic conditions and for heterotrophic organisms Bell et al (1980) used a spread plate method with a glucose-nitrogen minimal medium, supplemented with casamino acids, incubating at 5° and 20° and counting colony forming units (CFU) after 1 and 2 weeks. Nuttall (1983) sampled the River Dee and spread diluted samples onto a casein-peptone-starch medium incubtating for 21 days at 10°, the annual mean temperature of the river. Jones (1980) points out that most published work in freshwater microbiology reports results on plankton but benthic populations are often larger and more active. Problems are encountered with freeing organisms associated with particulate matter in sediments. Webster (1981) examined the microbial ecology of deposits of detritus and macrophyte vegetation from the River Thames. Samples were homogenised and diluted in a gelatin-ringers solution before being spread on a variety of complex media, which were selective for different organisms. Suberkropp and Klug (1976) cultured bacteria from leaf litter after homogenisation in cold filter-sterilised stream water, on a peptone-yeast extract-glucose agar for 1 week at 15°.

Apart from the aerobic heterotrophic organisms, there are other groups which require specialised cultural techniques. These include anaerobic heterotrophs from mud surfaces and deeper sediment samples, methane producers and photosynthetic bacteria (Collins 1963). Other autotrophic microbes include nitrifying organisms and sulphur-oxidisers. Gooday (1979) provides a comprehensive list of bacterial genera important in microbial ecological studies including those present in water but of animal origin which are facultatively anaerobic and have higher optimum incubation temperatures than indigenous aquatic microbes.

Having obtained viable cultures of as wide a variety of bacteria as possible; identification consists of a battery of morphological and physiological tests, following schemes such as those proposed by Shewan et_al (1960), Bonde (1966) and Straskrabova (1973). Nuttall (1982), examined dominant bacterial types according to Gram staining, colony appearance including colour, cell morphology, motility by hanging drop, oxidase production and production of fluorescent pigment on selective media.

Most investigators (Hopton, 1970; Suberkropp and Klug 1976, Takii, 1977; Bell et_al. 1980; Nuttall, 1982; subjected pure cultures to several biochemical tests including: ability to grow on different carbon sources; use of inorganic nitrogen sources; growth at different pH and temperature regimes; hydrolytic activity toward added casein, gelatin, starch, Tween 80; production of catalase, sucrose, phosphatase; and differentiation between oxidative or fermentative action with or without gas or acid production on a glucose medium.

Qualitative information on microbial types is complemented by measurements of bacterial numbers. Techniques for estimating microbial numbers and biomass have been reviewed and summarised by Jones (1979). Discussion is presented on pretreatment of samples, direct counts on membrane filters or in counting chambers, counting of viable organisms and the estimation of biomass by such factors as ATP content. Counting chamber methods, developed from counting other particles such as blood cells, are detailed in standard texts. (Norris and Ribbons 1969-1976).

Membrane filters of differing materials have been commonly used for direct counts of stained or unstained material (Jannasch, 1958), and the sensitivity of the method has been enhanced using fluorescent stains (Jones and Simon, 1975). Many workers have used the fluorescent staining technique for a wide range of water and sediment samples (Pomeroy and Johannes, 1968; Suberkropp and Klug, 1976; Hobbie et al. 1977; Goulder et al. 1980; Nutall, 1982).

Viable organisms have been enumerated by spread or pour plate techniques. These plates may also be used for identification purposes. Routine sanitary bacteriological counts use membrane filtered samples incubated in contact with suitable nutrient media. (Lin, et al. 1974; Anon, 1983).

Biomass estimation is usually carried out in studies of microbial metabolic activity (Iturriaga 1979). ATP is frequently measured (Holm-Hansen and Booth, 1966), with complementary measures such as DNA (Holm-Hansen, et al 1968), or chlorophyll (Vollenweider, 1974). The subsequent use of conversion factors (Winberg, 1971), allows interpretation of their chemical data in biological terms.

Rivers and Lakes being open environmental systems would be expected to have a wide range of micro-organisms either growing and multiplying there or present because of input from land or atmosphere. Collins (1963) screened 1000 cultures from lakes and flowing waters, finding commonly the genera: Pseudomonas, Flavobacterium, Alcaligenes, Chromobacterium, Micrococcus and Achromobacter now Alcaligenes (Hendrie et al. 1974). Suberkropp and Klug (1976) listed major and minor genera found in stream

water and associated leaf litter. Major genera were Flavobacterium, Flexibacter, Pseudomonas, Acinetobacter, Chromobacter and the minor genera were Serratia, Alcaligenes, Bacillus, Cytophaga, Sporocytophaga and Arthrobacter. Of the Pseudomonad species half were fluorescent. The Pseudomonad species were the commonest non pigmented bacteria. Optimum growth temperature was 18° to 22°. The coloured colonies ranged from yellow to orange and pink. Carotenoid pigments were characteristic of Flavobacterium and Flexibacter bacterial types.

Bell et al (1980) took a numerical method of taxonomic analysis by calculating similarity coefficients and clusters of isolates by the method of Sokal and Michener (1958) for heterotrophic bacteria in the rivers Meduxnekeag and Dunbar in Canada. Their findings as to bacterial types are tabulated below (Table 1.1) based on 6 samples for each river; 3 winter and 3 summer containing 100 isolates per sample selected at random.

The predominant saprophytic bacteria found by Sugita (1982) in the Edo River in Japan included Acinetobacter, Pseudomonas, Moraxella, Flavobacterium and Vibrio-Aeromonas and were distributed evenly both vertically and horizontally. Bacillus and Clostridium species were found seasonally in anoxic sediments with the aerobic Bacillis assumed to originate from the water column.

Bacteria commonly isolated by the methods most frequently used seem to be predominantly, (greater than 90% of types), rod shaped, gram negative aerobic and oxidative. Less than 1% are coccoid.

TABLE 1.1

Number of individuals of each genus expressed as percentage of total.

Number of species in brackets.

GENUS	WINTER		SUMMER	
Pseudomonas	71	(9)	65	(9)
Alcaligenes	*1	(1)	9	(2)
Aeromonas	5	(1)	4	(2)
Chromobacterium	5	(2)	*1	(1)
Plesiomonas	1	(1)	0	(0)
Flavobacterium	5	(3)	6	(1)
Cytophaga	10	(3)	7	(3)
Flexibacter	*1	(0)	4	(1)
Escherichia	0	(0)	*1	(1)
Enterobacter	0	(0)	*1	(1)
Klebsiella	0	(0)	*1	(1)
Acinetobacter	2	(1)	2	(1)

TYPE		
Obligate aerobes	74	70
Facultative anaerobes	15	12
Gliding bacteria	11	11
Enteric bacteria	-	3
Others	-	4

* less than

Footnote:

These results clearly indicate the dominance of the bacterial flora by the Pseudomonads and the suggested seasonal effects for genera such as Acaligenes, Chromobacterium and enteric bacteria, which tend not to be isolated in winter.

Apart from the genera described above, which tend to be psychrophilic, non-pathogenic and indigenous to the aquatic environment, there has been wide ranging investigation of bacteria classed as indicators of the presence of human and animal pathogens.

The recent report: "The Bacteriological Examination of Drinking Water Supplies" Anon (1983), explains the rationale of searching for organisms that are indicative of faecal pollution rather than attempting to isolate and count potentially low numbers of individual pathogens. Indicator organisms should be present in large numbers in sewage; essentially absent from other sources of pollution; be unable to multiply in natural waters and yet be easy to isolate, identify and count. Organisms fulfilling enough of these criteria to qualify as indicators include Escherichia, total coliforms, Salmonella group, faecal Streptococci, Clostridium perfringens and Pseudomonas aeruginosa, which although it can grow in natural water is a useful indicator in treated water of gross bacterial deterioration.

Klebsiella species are commonly found in polluted waters with Klebsiella pneumoniae reported as the principal coliform in a pulp mill wastewater, Hendry (1982). Non tubercular Mycobacterium species were isolated in the water column of the Morla River in Ital (Romanelli, 1980) and Aeromonas hydrophilia, which can be pathogenic is relatively common in warm waters Domokos (1981).

Of concern from a public health point of view is the possibility of proliferation of pathogens in natural waters. Daubner (1981) found that wastewaters from dairies, breweries and meat processing factories supported regrowth of: E Coli. Pseudomonas aeruginosa and Salmonella species. Singleton (1982) concluded that Vibrio cholerae could be induced to grow under the ambient conditions of organic nutrient and salinity commonly encountered in estuaries.

Efforts have been made to use bacteriological quality as an indicator of water pollution. The work of Lin et al (1974) concentrated on the bacterial quality of the Spoon River in Illinois in relation to Environmental Quality Standards set by the Illinois Pollution Control Board but suggested incidentally that faecal coliform to faecal Streptococci ratios enabled a distinction to be drawn between bacterial pollution from human as opposed to animal sources. They also stressed the need to take account of non-point sources of pollution such as agricultural run-off when assessing bacterial quality of surface watercourses.

Because of the physiological versatility of many of these organisms and the range of habitats presented by natural waters, it is likely that future studies will amplify the role of bacteria in the natural history of rivers and lakes.

1.1.3 Bacteria Associated with Sewage Treatment

Bacteria make up the major part of the biomass in all stages of biological sewage treatment and are responsible for most of the organic matter and energy transformations in the process.

Qualitatively the bacterial flora has links with the human, animal and industrial origins of sewage and with the natural or indigenous genera of bacteria found in rivers and soil. Conversely in rivers or lakes receiving an input of sewage the microbial flora will have a significant number and variety of active bacteria that originate from sewage treatment plants (Hynes, 1960; Yoshikura, 1981). It is to be expected that microbial processes encountered in the diverse microhabitats found in rivers and lakes would be identified again in biological sewage treatment plants, which are part of the same overall open system of natural water flowing through channels, retention cells and over surfaces.

Sanitary sewage originates from human voidings and the principal bacterial input would be of human origin. Additional input could derive from organic industrial waste water. Relevant processes could range from textile manufacture to fellmongery and tanning, both of which involve bacterial action on animal skins, to fermention industries such a pharmaceuticals and brewing and perhaps farm drainage such as cattle or pig slurry. This range of microbial systems would be likely to result in large numbers of a wide range of bacteria in a mixed domestic and industrial sewage. Table 2 provides information on counts of bacteria in sewages and effluents.

Counts reported in Table 1.2 illustrate the difference between total and viable counts, the relatively small proportion of coliforms in the total of viable organisms and the suggestion of inhibition of bacteria in industrial sewages. Sewage treatment whilst producing large amounts of microbial biomass has the effect

Table 1.2

Bacterial Numbers in Sewages and Sewage Effluents

Site	Counts Numbers/ml (* geometric mean)			Ref
	Total	Viable: (22° plate)	E Coli	Presumptive (Coliform)	
Industrial sewage		1.8×10^6	3.9×10^5	5.6×10^5	Harkness 1966
Domestic sewage		7.6×10^7	3.6×10^6	7.0×10^6	Harkness 1966
Settled sewage	5.6×10^8*	6.3×10^6*			Pike 1975
Activated sludge liquor	5.9×10^9*	4.9×10^7*			Pike 1975
Secondary effluent	5.4×10^7*	1.1×10^6*			Pike 1975

of causing a large reduction in bacterial count as treatment proceeds. Bacterial counts in sludge and anaerobic stages of treatment are difficult to evaluate because of the wide ranges of suspended matter, redox regime, organic content and nutritional requirements of anaerobes. Toerien et al (1968) reported 3 to 5 x 10^4 sulphate reducing bacteria per ml of raw sewage sludge. Proteolytic bacteria were present in digesting sewage sludge to the extent of up to 7 x 10^4 per ml (Harkness, 1966). Crowther and Harkness, (1975) quote a figure of 10^6 to 10^7 coliforms per ml of sludge suggesting that total anaerobic counts would be much higher.

Sewage treatment processes aim to produce an aqueous effluent of an organic and toxic character that will not cause a quality deterioration in the water body to which it is discharged. Conventional purification proceeds by a combination of particle settlement to a sludge, the maximising of heterotrophic consumption of organic matter by microbial biomass and the recovery of surplus biomass also as a sludge.

Changes in the bacterial flora of the sewage as it progresses through treatment reflect the community response to the sewage microenvironment (Gaudy and Gaudy, 1966). Of particular sanitary significance is the reduction in the numbers of pathogenic organisms. Kabler (1959) reviewed reports of removal of pathogenic micro-organisms in sewage treatment processes, concluding that whilst there was a marked reduction in numbers of pathogenic enteric bacteria and viruses they were not completely eliminated.

Identification methods for bacteria in sewage and sewage treatment processes are essentially the same as those methods for bacteria found more generally in aquatic environments. More attention has been paid to the problem of extricating bacteria from the different particulate matter encountered in sewage; for example faecal matter, slimes in sewers, primary and secondary sludges, percolating filter or bacteria bed films, activated sludges, zoogleal slimes, sewage fungus growths and bacterial aggregations or flocs. Pike et al (1972) reviewed methods of releasing bacteria from activated sludge flocs and they detailed their own trials using mechanical homogenisers and ultrasonic vibrators with Ballotini beads. They settled on an ultrasonic treatment as giving minimal lethal effect and found that counts were more affected by the pH of sample diluent and recovery medium rather than by ultrasonication or treatment with enzymes or colloidal peptizing agents to break up flocs.

Culturing technique has been noted as an important predetermining factor in identification protocols. Media have ranged from simple nutrient agar (Allen, 1944), to filter sterilised sewage agar with added polypeptone (Sakurai, 1967), which takes account of the low nutrient levels normally experienced by aquatic bacteria, to a reported general purpose medium of casitone, glycerol and yeast extract recommended by Pike et al (1972).

Two differing approaches to the task of identification of bacteria recovered in pure culture from sewage samples are typified by the work of Witthauer (1980), and Lighthart and Ogleaby (1969).

Witthauer chose to consider those bacterial genera reported more than twice in reviewed literature. A determination key was developed from those genera, based on cited reports, but taking care not to contradict descriptions in Bergey's Manual 8th edition (Buchanan and Gibbons, 1974). Branched keys were devised from the results of a battery of cultural, morphological and physiological tests. Two principal keys were used based on the reaction of bacteria to the gram staining procedure. Their object was to assign each isolate to a genera and hence relate operation of sewage treatment in model plants to observed changes in bacterial flora.

Underlying the work of Lighthart and Oglesby, (1969), was an assumption that a detailed knowledge of the structure and dynamics of microbial populations is essential for optimizing design and operation of sewage treatment plants. It appears that only recently has any progress been made in this direction with attempts to control the microbial inoculum of sewage treatment (Winkler, 1983). In order to realise this objective the authors evaluated large numbers of isolates using an array of as large a number as possible, in this case sixty, of morphological, biochemical and physiological tests. Known organisms were also subjected to the test protocol. Test results were used to measure similarity between organisms, which were then clustered according to principles of numerical taxonomy outlined by Sokal, (1963). Groups of organisms could then be compared with known organisms or, as was intended by the Authors, taken as functional units for design or operation purposes.

1.1.3.1 Bacteria of Sewage

Sewage has a widely fluctuating bacterial quality. Harkness (1966) reported monthly bacterial counts in crude sewage at two sewage treatment plants in Birmingham, one receiving an industrial sewage, the other an essentially domestic sewage for 1965-66. There appeared to be a ten-fold increase in numbers of both presumptive coliforms and plate count on nutrient agar at 20°C in the domestic as compared with industrial sewage. Harkness also reported the presence of faecal Streptococci and E Coli. Lighthart and Oglesby's clustering technique split bacteria from raw wastewater into three broad groups: Group I similar to faecal forms such as E Coli; Group II similar to Pseudomonas aeruginosa and a non defined group III which gave white colonies capable of growth at 35°C, anaerobic growth at 20°C but few of the other common features. Pike (1975) added to the list of genera the obligate anaerobic Bacteriodes species and facultative anaerobes such as Streptococcus and Lactobacillus, which individuals would not be expected to take an active role in the aerobic stages of sewage purification.

1.1.3.2 Bacteria of Aerobic Biological Treatment

Following the conveyance of sewage to the treatment works the preliminary sedimentation stage causes reduction in the numbers of coliform bacteria. (Ware and Mallon, 1956), and simultaneously the start of the development of the characteristic flora of conventional treatment. Often secondary biological sludges such as a surplus activated

sludge or humus solids, are returned to the incoming flow of sewage to be settled with raw sludge and this would be an effective inoculum of indigenous bacteria.

Much work has been carried out to identify the bacteria present and active in the two conventional methods of biological treatment, which are activated sludge and percolating filters (Butterfield and Wattie, 1941; Allen, 1944; James 1964). A typical result of this and more recent work (Witthauer 1980) is that the predominant bacteria are gram negative aerobic rod types. The variation and composition of the waste seems to have little effect on the bacterial flora with the following genera recovered in large numbers: Pseudomonas, Achromobacter (Alcaligenes) and Flavobacterium. Some treatment plants appeared to have flora dominated by either Pseudomonas or Achromobacter/ Flavobacterium. Lighthart and Oglesby (1969) refined these groupings by linking Flavobacterium with Cytophaga and Pseudomonas with Aeromonas-Vibrio.

Rationalisation of taxonomic grouping based on more recent work has meant that organisms formerly afforded species status have been either redefined and included with other genera, or noted as unsupported reports or uncertain genera. Thus the Achromobacter genus was changed in Bergey's 8th Edition (Buchanan and Gibbons 1974) to either Alcaligenes or Acinetobacter. The cluster analysis approach of Lighthart and Oglesby (1969) is more amenable to such u[illegible]stings because broad groupings are used.

There has been debate about the status of the genus Zoogloea which was reported by Butterfield (1935) and Dias and Bhat, (1964) in activated sludge and in percolating filters by James (1964). Debate centres on whether, the type species which was described by Itzigsohn (1868) Zoogloea ramigera as a gram negative rod, occurring in gelatinous slimes in filters or in sludge flocs in activated sludge, is a true species or whether the properties of floc or zoogloeal slime formation are simply responses of an essentially non fluorescent Pseudomonas to cultural conditions as yet physiologically ill defined in the sewage environment. Crabtree and McCoy, (1967) and in Bergey's 8th Edition (Buchanan and Gibbons, 1974) propose the validity of the concept of a distinct Zoogloea ramigera even though other authors (Uns, 1971) argue about neotype strains.

Linking the man made environment of a sewage treatment plant with natural waters is the phenonmenon of sewage fungus. In his review paper Curtis (1969) concludes that sewage fungus, or in its equivalent terminology Abwasser Pilz (Tiegs, 1939) or heterotrophic biocoenosis (Wuhrmann, 1954), is a sensitive indicator of organic pollution in flowing waters. Filamentous organisms (bacteria, protozoa and fungi) make up an attached matrix within which other organisms from motile bacteria to protozoa, diatoms and higher invertebrates find a suitable habitat. It may be viewed as an assemblage of organisms comparable to bacteriological filter slimes or activated sludge that is exploiting a habitat niche. Curtis' 1969 survey of sewage fungus in the UK for the former Water Pollution Research Laboratory showed that of the bacteria present; Sphaerotilus natans was in a predominant position with

Zoogloea. Flavobacterium and Beggiatoa species in significant numbers or amounts. Again the status of Zoogloea is questioned despite its obvious active oxidative but non proteolytic metabolism. Organisms such as Sphaerotilus may show a zoogloeal type of habit under conditions of low flow and high organic carbon content.

A group of bacteria which Witthauer (1980) reports as probably of importance in some waste treatment systems are the Coryneforms. Arthrobacter is the common genus. This genus was reported by Adamse (1968) as a dominant bacterium in an activated sludge plant treating dairy waste. In older cultures and probably in nutrient poor natural aquatic environments Arthrobacter exhibits pleiomorphism and would tend to be coccoid. Other genera such as Micrococcus may therefore have been incorrectly recorded instead of Arthrobacter.

1.1.3.3 Bacteria of Anaerobic Treatment

Prior to and during the bio-oxidative treatment of sewage there is the production and separation of sludges rich in carbohydrates, lipids and proteins. Anaerobic conditions are rapidly established in these sludges. Treatment is based on encouraging fermention, usually at either ambient temperature or often in a mesophilic range at about 35° Celsius. Harkness (1966) and Crowther and Harkness (1975) review work on the bacteriology of sludge digestion, finding that comparatively little work has been reported. Organisms found to play an active role in anaerobic breakdown of organic matter, initially by hydrolysing carbohydrate, lipid and

protein polymers, are: Sarcina, a gram positive coccus, Bacillus and Clostridium. (Cookson and Burbank, 1965; Toerien, 1970;) a Streptococcus (Buck, et al. 1953) and Bacteroides (Hobson and Shaw 1971). These organisms would be expected in the anaerobic habitat of sewage sludges.

It is likely that organisms capable of facultative anaerobiosis present normally in sewage for example Pseudomonas, Micrococcus may play a minor role in the sludge treatment system. (Toerien, 1967).

Of major importance from an operational point of view are methanogens which significantly reduce the organic content of the sludge, particularly the lipids. This renders the sludge stable, easier to dewater or more acceptable for agricultural land disposal.

In reviewing and discussing the bacterial flora of natural waters and such related environments as sewage treatment plants and probably even soils, it would be desirable to rationalise the groupings of organisms with the objective of suggesting relationships between groups. Many genera of bacteria can be isolated from aquatic environments with major groupings such as Pseudomonas, Flavobacterium, what has been frequently termed the Achromobacters and the Enterobacteriacae. Minor groupings include Coryneforms such as Arthrobacter; Cytophaga, Vibrio and gram positive sporeformers.

There is a risk of over complication when, in adopting an analytical approach, it becomes apparent that groupings need to be subdivided; for example the Achromobacters are now considered more accurately described as Alcaligenes or Acinetobacter. Bergey (Buchanan and Gibbons Ed. 1974) however, does not link these two genera very closely. Alcaligenes has the status of an uncertain genus attached to the Pseudomonanads or gram negative aerobic rods and Acinetobacter as a gram negative Coccobacillus.

Similarly there is continued uncertainty about the validity of the Flavobacterium group in that it relates to the gram negative facultative anaerobic rods, which includes the Enterobacteriaceae, and seemingly at the same time it is linked with the gliding bacteria such as the Cytophaga.

To maintain a simplicity here it may be better to restrict the attempt to reach a precise identification and limit discussion to biological factors such as physiological adaptation to the nutrient status in natural waters, aerobic or facultative capacity, temperature adaptability and biochemical potential.

1.1.4 Processing of Material in Aquatic Systems

1.1.4.1 Role of Particulate Matter

Watercourses act as conduits for water falling as precipitation. This water interacts chemically with the atmosphere, rocks and associated soils, to produce an aqueous solution of variable

composition. By virtue of the kinetic energy of the flowing water, rivers shape the landscape by the process of erosion, loading the channel with particulate matter. Leopold (1964) aptly sums this up by calling rivers "gutters down which flow the ruins of continents".

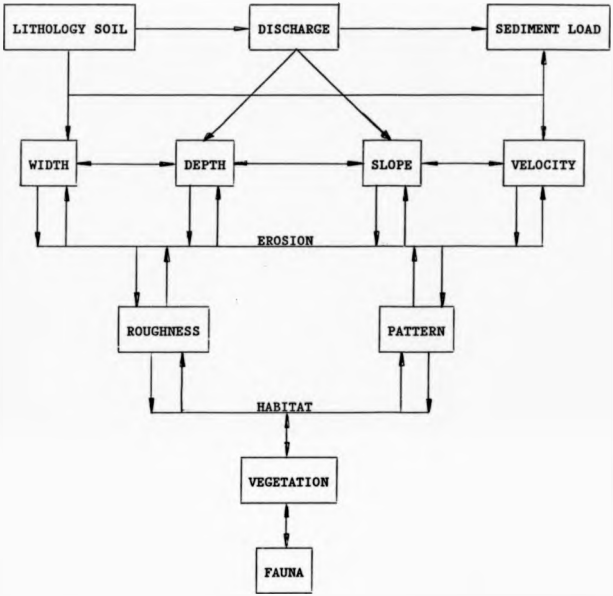
Geomorphological studies aim to describe the processes responsible for the creation of river basins. Morisawa (1975) suggests the existence of feed back loops in river systems moderating the evolution of channel character. Should these loops have a significant potential, they could be given an added biological dimension as indicated below, figure 1.1. Thus the type of vegetation is in part determined by all the physical attributes of a river and can at the same time by altering the roughness of a channel directly and indirectly affect channel slope, velocity, discharge and hence sediment load.

This view conflicts with that of Wuhrman (1972) who views rivers as continuous plug flow fermenters having part of the biomass free in suspension, part fixed as epibenthic growth and part in sediment or benthic biomass. He considers there to be no feedback to upstream reaches.

As an example of the likely importance of the above physical characters in habitat creation the work of Savini and Bodaine (1971) indicates a logarithmic rate of velocity decrease with distance from the streambed. Colonisation of such a velocity profile would be expected to be selective as to type and numbers of organisms adapted to different current regimes and

Figure 1.1

Physical Factors Affecting River Channel Morphology
After Morisawa (1975)



particularly if fluidized bed conditions arise in looser sediments.

On a macro scale Morisawa (1985) reviews reports of suspended solids loads from large river basins. Holeman (1968) quotes a range of 107 to 2913 tons/km^2 between the Mississipi USA and Yellow River, China. This compares with a notional figure of 10 tons/km^2 for the River Severn in 1985 (Severn Trent Water Authority). Catchments with comparatively microscale areas can yield a wide range of suspended solids loads as reported by Wolman (1967). Table 1.3.

Solid matter transported by rivers can be divided into two main types: the bed material and the wash load. (Ackers 1985).

Sediments are further characterised by Einstein (1972) taking into account their size ranges and the ability of the flow to move the particles. Coarser paricles have sufficiently high settling velocities to become part of the bed material, where all particle sizes but the smallest are well represented. The fine particles would be included in the wash load; which moves at roughly the speed of the flow. Bed material would tend to move only at high flow rates. Hence heavier particles may take 50 to 100 years to travel a distance equivalent to that moved by wash load particles in one flood. Wash load tends in consequence, to be a function of catchment nature; whereas bed material loading is a function of flow rate. This has a consequence for the potential of a river to support different flora and fauna and may be significantly affected by anthropogenic effects such as sewage input or urbanisation.

Figure 1.2

Partitioning of Solid Matter in Rivers (after Ackers [1985]

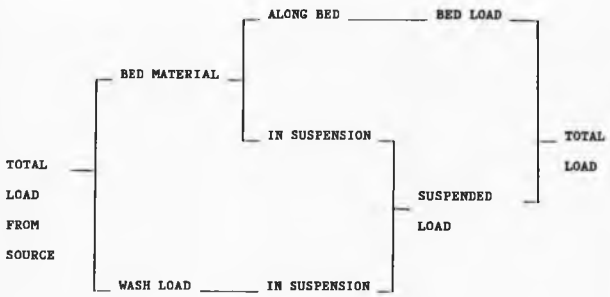


Table 1.3

After Wolman (1967)

Sediment Yield of Different Catchments

Drainage Area (km^2)	Land Use	Sediment Yield (tons/km^2/year)
0.006	Construction site, soil stripped	127,000
6.4	Urban area	49
19.2	Forested area	10
785	Agricultural	735

Table 1.4

Particle Sizes of Dried Deposits (with organic matter destroyed)

(Kennedy 1977)

Particle type	Mean Diameter (mm)	% of total particles	
		Fast Flowing River	Slow Flowing River
Coarse Sand	0.2 – 2	88.6	1.5
Fine Sand	0.002 – 0.2	9	31.3
Silt and Clay	0.002 – 0.02	1.6	41.9
$CaCO_3$ & Soluble Salts	less than 0.002	0.8	25.3

Particle size ranges and nature have been the subject of much work particularly in relation to sediments. Hamence (1967) analysed muds from various clean and dirty rivers, lakes and estuaries. Grouping together the clean waters, the moisture content and percentage loss on ignition of the dried solids were 71% and 17% respectively. Equivalent figures for polluted waters were 81% and 27% respectively. Both sets of averages had wide variability but do illustrate the high water retentive character of sediments and the enhanced organic content of polluted systems. Table 1.4 based on Hamences' results shows the particle size ranges and mineral constituents of particulate matter from river sediments.

Jones (1980) investigating differences in the microbiology of littoral and profundal lake sediments carried out wet sieving of samples in four size groups from 45 um to 500 um. His results for dry weights of particles complement Hamences' above showing that lake sediments are similar to slow flowing rivers having a high proportion of particles less than 100 um. Particulate matter separated by Jones (1980) had had no pretreatment to remove organic matter which was present to a similar extent in all fractions. In both littoral and profundal sediments bacterial counts were highest for the small particles less than 45 um which were probably silts and clays. Both Authors found similar organic contents for sediments at around 5 - 15% by weight. Nitrogen and phosphorus contents of organic solids were 5% and 0.25% respectively (Hamence, 1967). In contrast dry sewage solids had a typical organic content of 60% with nitrogen up to 10% and phosphorus 5% of organic matter. Therefore the presence

of sewage solids in a watercourse could significantly alter the nature of natural sediments.

Average figures for mineral matter, organics and nutrients do not allow description of the dynamic nature of the relationship between sediments and water column. Hill (1982) showed that phosphorus was absorbed by stream bed sediments in substantial amounts under low flow conditions. Clay minerals and fine solids which constitute wash loads move in and out of the particulate phase of rivers and lakes. Kaolinite clays tend to settle more readily whereas montmorillonite clays are flocculent with high water contents with the result that microbial habitats would be different for waters similar in other ways for example temperature and biochemical oxygen demand where mineralogy of wash loads varied (Einstein, 1972).

Ecological analysis of habitat includes budgetting the energy and material flow through the system. For rivers, this analysis is rendered difficult because of the dynamic nature of the fluvial system. Any description of the material budget of a river would consider inorganic components such as sands and clays and the more biologically significant organic matter. An annual organic matter budget for a short length of unpolluted fourth order watercourse was formulated by Fisher (1977). His work identified pathways or vectors for organic matter transport including "meterologic", precipitation or wind for example leaf fall, "hydrologic", dissolved or particulate load in flowing water and "biologic", primary production, respiration or intrasystem processing and immigration. Organic matter in the system was categorised

Table 1.5

Organic Matter Budget for Clean River (after Fisher, 1977)

Organic Matter Type	Size	kg/m^2 year	
		Import	Export
Coarse particulate matter	** 1 mm	0.49	0.19
Fine particulate matter	* 0.1 μ * 1 mm	7.49	6.83
Dissolved organic matter	** 0.1 μ	22.23	22.61

* Greater Than

** Less Than

according to sizings that had a biological significance. Table 1.5 modified from Fisher's work summarises the major features of the organic matter budget.

Coarse matter is principally leaf litter and hence seasonally abundant; if not trapped it is rapidly exported from the system. Fine matter is proportional to stream discharge including the antecedent flow which is a measure of availability of particles in the stream bed. Plots of fine particulate organic matter against flow reveal two populations of figures split seasonally, June to November and December to May. In contrast dissolved organic matter is inversely proportional to discharge again with a seasonal pattern evident from plots of organic matter versus flow. As to primary production and respiration Fisher (1977) found that 90% of total production was associated with periphyton and for respiration the predominant heterotrophic contribution was by micro-organisms not macroinvertebrates. This heterotrophic activity related to the fine matter which predominated in the 24% of total organic matter that was particulate. No explanation was given for the apparent failure of the system to utilise more of the dissolved organic matter; this may be a function of residence time in that dissolved organic matter might be expected to pass more rapidly through the system.

Rivers and lakes in unpopulated areas may well represent more natural background ecosystems but many rivers of practical importance both for water supply or as health risks are also carriers of sewage or contaminated land run-off. Inputs of

this type will add to the already complex picture painted by Fisher above.

Some study has been carried out on what have been termed non settleable solids by Zanella et al (1978) and Friant, et al (1980).

Zanella et al (1978) worked on paper mill effluents after biological treatment. Solids had a size range of 1 to 16 µm with 80% of particles in the 1 to 2 µm range, which includes bacterial cells, cell debris and inorganic coating materials. Larger sizes greater than 2 um included small bacterial flocs, fibres and some protozoa. Less than 10% of the particles numerically in counts, accounted for up to 60% of the volume of particulate matter. These particles could completely support the growth and multiplication of macroinvertebrates such as Hydropsyche species (caddis fly) and microcrustacean Daphnia species. Chemical analysis showed that the solids ranged from 20 to 50% organic carbon and 20 to 30% Protein. ATP measurements linked to factors suggests that between 3 to 45% of the biological material was alive with overall viability 22%.

Friant and co-workers investigated various treatment plants including domestic sewage and industrial effluents from paper mills and textile manufacture. Non-settleable solids were counted by particle counting equipment for the range 0.3 to 5800 µm or by scanning electron microscopic examination. Solids were found to be agglomerated into small flocs of bacteria with associated colloidal matter, virus particles,

flagella and cell debris. It was concluded that secondary or complete biological treatment seemed to generate these non-settleable particles but the profile of the particles varied from plant to plant. One plant at Green Bay Wisconsin had particles less than 2 μm dominated by flagellated bacteria with abundant amorphous material equal to or less than 1 μm. There were some large rod shaped bacteria up to 6 μm and flocculant material between 5 to 8 μm. A sewage treatment plant at Meehopany had many dispersed bacteria up to 3 μm, rod shaped bacteria between 3 to 6 μm, chains of bacteria up to 12 μm and flocs in the range 5 to 10 μm. Work was reported on the fate of these particles upon discharging to receiving watercourses, which showed that labelled bio solids were taken up by macroinvertebrates (Anon, 1978). More emphasis was placed upon their effects on fish stocks, algal growth and macroinvertebrate communites.

In assessing the role of bacteria in organic matter processing in rivers it may be that greater detail on the make up of components of fine particulate organic matter to complement bacterial identification would help in explaining other biochemical and physiological characteristics.

1.1.4.2 Role of Soluble Matter

Particulate matter shares the common matrix of water with matter in solution or pseudo-solution. Both are intimately involved in a cycle mediated by physical, chemical and biological processes. Water chemistry is a wide subject but mention is made here of the constituents of significance to bacterial growth and physiology.

Levels of total carbohydrate, protein and amino nitrogen in the interstitial water of littoral and profundal sediments were reported by Jones (1980). Carbohydrates in mg/l were 2.5 and 4.5; proteins 0.4 and 0.56 and amino nitrogen in micromolar glycine equivalents were 1.2 and 1.3 in the littoral and profundal sediments respectively. These concentrations would not be expected in the water column on a gross scale, but may exist in the micro-environments associated with sedimenting particulate matter where heterotrophic and autotrophic activity is enhanced.

Earlier work (Peterson, et al, 1925) reported much lower levels of amino acids in Lake Wisconsin: Tryptophane 5 to 14 µg/l; Tyrosine 8 - 17 µg/l.

In water where conditions make significant primary production possible Mann (1972) showed that chlorophyll a or phytoplankton was nearly 30% of the annual mean dry weight of solids in the River Thames above the Kennet confluence. The Kennet in contrast had a higher content of organic matter but phytoplankton in this case constituted only 5% of the total. This was attributed to the sewage content in the Kennet downstream of the Reading sewage outfall.

Sewage pollution of rivers has stimulated study of chemical character and change of character with treatment. Eickert and Hunter (1969) partitioned TOC and particles in raw sewage and activated sludge plant effluent as summarised below in Table 1.6.

Table 1.6

Partitioning of Organic Matter in Sewage (after Rickert and Hunter, 1969)

Component	Particle Size	Raw Sewage	Effluent
Dissolved	**0.001 μm	42%	69%
Colloidal	0.001 - 1 μm	11%	6%
Supracolloidal	1 - 100 μm	20%	25%
Settleable	* 100 μm	27%	-

** less than

* greater than

This organic matter in sewage effluent was further characterised by Manka et al (1970) in Table 1.7.

Table 1.7

Characterisation of Organic Matter in Sewage Effluent (after Manka, 1970)

	% of Total Chemical Oxygen Demand	
Component	Filter Effluent	Activated Sludge Effluent
Protein	21.6	23.1
Carbohydrate	5.9	4.6
Ether extractable	16.6	16.0
Tannins and lignins	1.3	1.0
Fulvic acid	25.4	24.0
Humic acid	12.5	6.1
Anionic detergents	16.6	16.0

These effluents appear to be well stablised as evidenced by the high humic and fulvic acid contents. The anionic detergent content appears high and more carbohydrate may have been expected to be present. Both effluents despite being from different treatment processes seem very similar chemically.

In concluding it appears that microbial growth potential is determined by the existence of habitat which in turn is intimately bound up in the surface and particulate character of the aquatic environment. Of equal importance is the availability of organic matter and nutrients such as nitrogen, phosphorus and trace metals to drive the systems. Description of microbial physiology must take account of these factors.

1.1.5 Microbial Ecology

1.1.5.1 Introduction

Microbial ecology studies attempt to draw together information from various disciplines such as bacteriology, biochemistry, physiology, chemistry and hydrology in order to synthesise a model of the natural processes established in the system under investigation (Cairns, 1971). To understand the roles of bacteria in aquatic systems it is necessary to complement data from defined systems, which fail adequately to model complex natural systems (Heath, 1979), with statistical analyses of natural environmental data (La Riviere, 1972). Pike (1975) summarises the approaches as microscopic; to estimate total and viable cell numbers; cultural to identify organisms; metobolic to measure kinetic constants and substrate turnovers and energy budgeting to describe energy flow between trophic levels.

All natural water bodies have a catchment and the regime of the system will be determined to a greater or lesser extent on that catchment; such matters as the input of allochthonous organics or more basically the volume, flow rate and raw chemical nature of the water.

A more thorough knowledge of microbial ecology of aquatic systems helps in tackling problems of water pollution such a prediction of the fate and action of pollutants both organic and inorganic in rivers and lakes or optimisation of treatment processes to minimise polluting effects as economic costs.

1.1.5.2 Habitats

Aquatic systems can be seen to be compartmentalised. Spatial compartments are almost invariably included as a dimension to a study of microbial ecology. Barnes and Mann (1980) summarise these basic considerations starting with the water balance between oceans, with more than 95% of Earth's water, to lakes, with 0.02% to rivers with 0.0001%. Compartments include the pelagic, water mass or water column, benthos being the underlying sediments or rocks and the shallows or fringing environment. Rivers are dynamic flowing systems where upper reaches may be compared with rocky margins of lakes and silty lower reaches are analogous to lagoons or estuaries. Lakes could be taken as relatively static systems but may have large internal water movements or seiches, riverine inputs and vertical stratifications or convection currents caused by temperature gradient, which will all inject a dynamic element to these systems.

Superimposed on these macroscopic habitats are the distinctions between planktonic (floating) or nektonic (swimming) communities in the water mass. Both of these communities would be included in the seston, which is the total particulate matter present in the water, living or non living. Of considerable importance are organisms which grow attached to solid substrates. This community has been termed periphyton, aufwuchs or haptobenthos (Hutchinson, 1975). Sampling of this habitat is particularly difficult without disturbing the spatial and functional relationships inherent in it.

Energy flows through aquatic systems would be determined by the balance of primary production of organisms such as autotrophic bacteria, algae and macrophytes with additional allochthonous input from the catchment or sewage discharge, set against the attendant development of heterotrophic detritus food chains. Producers and decomposers can develop as communities in any of the habitats defined above. Detritus habitats are of particular importance when considering heterotrophic activity, where there is multiple repetitive processing of organic matter and where the bulk of organic detritus is non living and perhaps ten to a hundred times the amount of living matter (Pomeroy, 1979).

Microcosms have been used for studies of microbial ecology with some success but Dudzik et al 1979) points out the limitations of these experimental set ups. Serious size related problems include shallow depth preventing migration of

protozoa and causing sedimentation of algae, lack of higher trophic levels for example fish and dominance of side and bottom container wall effects which may be 10^4 times greater than in natural systems. Dudzick et al found that a close packed layer of cells 1 μm in thickness for a 1 m diameter deep tank gave 4 cm^3 of biomass per 1 m^3 of tank which is equivalent to the volume density of phytoplankton in a mesotrophic lake.

1.1.5.3 Attachment of Micro-Organisms

Solid-liquid interfaces have for some time been acknowledged as important sites of microbial activity; both for production but perhaps more importantly for heterotrophic activity. An increase in growth of marine bacteria in bottles was noted by Zobell (1943) and further enhancement was obtained on adding inert particles. Marshall (1976) has summarised findings on the effects of surfaces on microbial activity. Other authors separately report the greater uptake of dissolved organic carbon by epibacteria (particle associated) as compared to unattached bacteria, (Kirchman and Mitchell, 1982). Attached heterotrophic bacteria have a dominant role over planktonic forms in the self purification of shallow streams (Marshall, 1978). Particulate matter produced by the planktonic community is rapidly colonised by heterotrophic micro-organisms (Iturriaga, 1979). Joint and Pomroy (1982) studying heterotrophic organisms in the Bristol Channel found the highest activity in the most turbid regions. Bell and Albright (1981) reported that 60% of the bacterial biomass and heterotrophic activity in a turbid river was associated with

the suspended particulates. In continuous culture of river water, Hendricks (1974), found that natural populations of heterotrophic bacteria sorbed to glass surfaces and appeared to be more metabolically active than organisms in suspension.

Many authors (Collins, 1963; Pike, 1975; Nuttall, 1982) have reported the discrepancy of counts for total bacteria by direct microscopical counting methods compared to estimates of colony forming units. Estimates by Iturriaga (1979) of colony forming units range from 0.2 to 11% of total bacterial numbers as measured by epifluorescence counts. Explanation centres on variations in viability of individual cells under cultural conditions employed and the possibility of lost counts due to aggregation of bacteria on particulate matter.

Marshall (1978) describes the microbial succession on, for example a clean glass surface immersed in a natural aquatic system. There would be an immediate adsorption of a layer of organic matter; within an hour small rods attach at 90° by their cell ends secreting extracellular polymers within a few hours. Spiral, coccoidal and larger rods take up to 8 hours to attach and there would be growth and overgrowth of slower growing organisms such as the stalked Caulobacter and Hyphomicrobium up to 7 days. Finally grazing by protozoa starts followed by colonisation by diatoms or algae depending on the light and nutrient status. Knowledge of the detail of this process would help in understanding the macroscopic processes of sewage treatment which are intimately bound up in the establishment of microbial films. A contrary view is expressed by Pomeroy (1979) who suggests that there can be

more suspended than particle associated bacteria because particles would be heavily grazed by protozoa producing a "close cropped lawn" effect. Most particles would be refractory and would present bacteria with no advantage in being there.

1.1.5.4. Organic Matter Processing

Natural rivers and lakes or more artificial aquatic systems like sewage treatment plants provide habitats for a wide variety of producer and consumer organisms. Of interest from a pollution control viewpoint would be problem growth of photosynthetic organisms causing eutrophication and more generally the consequences of enhanced heterotrophic activity brought about by input of organic matter. This leads to ecological imbalance and hence prejudice to water resource utilisation. Microbial ecology studies seek to describe these processes with a view to improving management of the problems. Study starts with detritus food chains and the role of aquatic bacteria. Estimates of the importance of bacteria vary but Pomeroy (1979) suggests that bacteria degrade more than 50% of the phytoplankton primary production in marine communities. In systems such as wooded low order streams with little primary production but high rates of allochthonous input from for example leaf litter the role of bacteria would be pre-eminent. Similarly rivers or lakes receiving sewage or organic input from land drainage contaminated with farm drainage would have high levels of bacterial biomass present. Fenchel and Jorgenson (1977) comprehensively review the role of bacteria in detritus systems pinpointing their essential

features as: their ability to assimilate and utilise a wide range of organic substrates and inorganic nutrients, at low concentrations; this is related to their high surface area to volume ratio, the possession of hydrolytic enzyme systems that enable them to breakdown nutrient poor plant tissues and the ability to function effectively in anaerobic or aerobic environments.

In theory all substances enter the bacterial cell in a soluble form but the distinction between dissolved and particulate detritus is relevant. Dead and dying plant and animal tissues do release soluble organic compounds such as glucose, glycollate and amino acids (Webb and Johannes, 1967) but much organic matter is present in a gross solid form for example leaf litter, macrophyte debris and dead macroinvertebrates. Parsons (1963) found the proportions of dissolved organic carbon, particulate organic carbon and living biomass in the ocean to be 100:10:2. A dynamic situation exists between organic matter in suspension and in sediments (Steele and Baird, 1972).

Of considerable importance in the processing of detritus are organisms that shred the material and by excreting faecal pellets provide a microenvironment for establishment of heterotrophic bacteria. A similar situation arises when polysaccharide mucus of various chemical types produced by higher organisms or plants leads to aggregation and flocculation of small detritus particles to form new sites for attachment and growth of bacteria.

All processing of detritus whether as soluble low molecular weight organics or complex plant or animal bodies will involve conversion of polymeric material to simpler absorbable compounds. This conversion will require enzymes produced and secreted by the organisms associated with the material, thus occupying an important position in the overall organic matter processing system.

1.1.6 Microbial Physiology

Natural water bodies, be they flowing or comparatively quiescent as lakes, have a capacity to process autochthonous or allochthonous organic matter. A fundamental component of this processing is microbially mediated and the importance is stressed by several workers. Hobbie and Crawford (1969) found that natural bacterial assemblages assimilate and respire more than 50% of defined substrates at naturally occurring concentrations. Sewage treatment plants typically remove, by a combination of particle sedimentation, microbial assimilation and biomass production, 90% of organic matter in sewage as measured by biochemical oxygen demand. Similarly rivers can self purify added BOD.

As previously discussed the major genera of bacteria isolated from natural waters or associated with organic matter processing in sewage treatment processes or sediments comprise, Pseudomonas, Alcaligenes, Cytophaga, Flavobacterium, Acinetobacter, Chromobacterium, Micrococcus, Bacillus, Arthrobacter, Coliforms and Coryneforms (Suberkropp and Klug, 1976). Methods of isolation by selective culture and

screening techniques based on metabolism indicate the range of physiological versatility of waterborne bacteria. Nutrient agar has been a common starting point to find a suitable general purpose isolation medium for heterotrophic bacteria (Hopton, 1970; Pike, 1972) but suspicion that counts were not optimal led to refinement of media. Complex solid media were developed using minerals, carbon sources such as glucose and sometimes additional growth factors or nitrogen sources in the form of yeast extract and protein hydrolysates. Media without complex supplements would be considered minimal media (Collins, 1963). Other authors combined various organic substrates: peptone, tryptone, casein hydrolysates, starch, gelatine to make up complex complete media (McKinney and Weichlein, 1953). A common combination of casein hydrolysate, yeast extract plus glycerol was selected by Pike, et al (1972), as giving a maximum recovery of organisms from activated sludge. Lighthart and Oglesby, (1969) used a multiplicity of complex media including: Cytophaga Agar, activated sludge agar and Dias and Bhat (1964) used sewage agar in an attempt to maximise recovery of aquatic bacteria.

Specific bacterial groups can be selected for by the use of selective media Bennets agar will select Chromobacterium species (Keeble and Cross, 1977) or King's media will isolate fluorescent Pseudomonads (King, et al. 1954). It would appear that despite the anticipated low nutrient status in aquatic systems the indigenous organisms often require complex media for good growth. Media however do tend to be less concentrated than those used for cultivation of pathogenic organisms.

Biochemical tests carried out on pure cultures of bacteria isolated from natural waters facilitate identification by comparison with standard keys such as those developed by Bergey (Buchanan and Gibbons, 1974) or Shewan et al (1960) for Pseudomonas. Tests would seek to find the extent of utilisation of different carbon sources; ranging from acetate to ethylene glycol to many other intermediary metabolites such as ketoglutarate (Bell, et al. 1980). Carbohydrate utilisation is commonly tested using sucrose, lactose, starch, glucose, galactose and other less common hexoses, pentoses and polysaccharides. In a complementary way nitrogen sources are investigated ranging from ammonia and nitrate to amino-acids. Hydrolytic enzyme action is considered an important diagnostic feature with activities of protease, using casein or gelatine, lipase using Tween 80, phosphatase, amylase and urease commonly measured. Metabolic patterns are demonstrated by such tests as: Hugh and Leifsons indicator of oxidative or fermentative catabolism of glucose, presence of cytochrome oxidase by Kovacs reagent, hydrogen sulphide production and nitrate reduction.

The physiological manifestation of biochemical activity has been studied in terms of growth experiments at different temperature, pH or redox regimes; the morphology of colonial outgrowth for example the spreading habit of Flexibacter species or pigmentation in Flavobacterium. Motility is invariably checked as is reaction to gram staining and the production of endospores. (Lighthart and Oglesby, 1969).

The significance of observation of biochemical activity in pure cultures at the relatively high nutrient or substrate concentrations in comparison with the much more complex and environmentally variable natural habitats is difficult to determine. Straskrabova (1983) describes the effect of so called substrate shock when starving aquatic bacteria are placed in a glucose containing medium from a natural low nutrient aquatic situation. Short term effects included a loss of viability and there were longer term losses of biochemical properties including nitrate reduction, gelatine liquefaction and tributyrin utilisation. It is a reasonable hypothesis that an organism such as Pseudomonas would utilise a wide range of carbon sources known to be used in in-vitro tests in comparison to a Flavobacterium: but that the Flavobacterium would be present in situ where insoluble proteins were available as a nutritional resource probably denied to a Pseudomonad.

This problem of measuring heterotrophic microbial activity has been extensively studied in marine and freshwater environments in terms of assimilation of substrates by organisms (Zobell and Grant, 1943) and by considering mineralisation of radio-labelled substrates (Kadota, et al. 1966). A frequently used method (Crawford et al. 1974) is one based on observation by Parsons and Strickland (1962) that what is termed "relative heterotrophic potential", or the uptake of labelled substrates such as acetate and glucose followed the Michaelis Menten pattern of kinetics. Wright and Hobbie (1965) developed these methods by using ranges of labelled substrate concentrations to enable better estimates of maximum uptake velocity Vmax

and, by extrapolation, estimates of rates of uptake at the naturally occuring low level of substrate. Allowance can be made for loss of carbon-14 during metabolism by measuring CO_2 released (Hobbie and Crawford, 1969).

A consequence of assuming Michaelis-Menten kinetics is the presumption that the heterogeneous assemblage of bacteria in natural systems will all have similar kinetic constants, Vmax and Km. Williams (1973) has shown this presumption to be a drawback and Hall et al. (1972) demonstrated that in sediment studies this source of error introduced much variability into the results of kinetic experiments comparing sediments with overlying water rendering biological interpretation difficult.

Goulder, et al (1980) investigated heterotrophic activity in an estuary with variable levels of pollution concluding that glucose mineralisation potential Vmax was positively correlated with measures of organic pollution such as permanganate value and ashed suspended solids. Activity rates per bacterium were not positively correlated with organic matter suggesting that nutrient availability favoured increased numbers rather than increased specific activity. A marked seasonal fluctuation in Vmax values were found by Iturriaga (1979) in the sedimenting particulate matter in the Baltic Sea; with higher values in summer than winter. Heterotrophic activity was found by Jones (1980); to be higher in profundal (deep water) rather than littoral (shallow water) sediments in two English Lakes and to be correlated with the degree of eutrophication. Profundal sediments also had higher microbial numbers expressed as direct counts and higher

concentrations, in interstitial water, of available substrates such as carbohydrate, proteins or amino acids.

Other authors report a correlation of Vmax with temperature (Munro, et al. 1973; Carney and Colwell, 1976; Bell, et al. 1980; Nuttall, 1982).

Albright and Wentworth (1973) found that Vmax, correlates with bacterial count and suggested that observed ^{14}C glucose turnover would be a good indicator of the trophic state of rivers. Vmax could probably be used as an indicator of pollution levels in water bodies (Carney and Colwell, 1976).

Other factors influencing the physiological requirements of the organisms responsible for processing organic matter in aquatic systems include the carbon to nitrogen to phosphorus to sulphur ratio of food sources. Hattingh (1963) reports that high carbon to nitrogen and carbon to phosphorus ratios in sewage determines the bacterial species make up in bulking activated sludge, which is characterised by extensive growth of filamentous bacteria. The same high carbon to nutrient ratios are stated to favour flocculation in strains of Klebsiella. (Duguid and Wilkinson, 1953).

Mixed substrates in activated sludge plants were investigated by Painter, et al (1968) who found that glucose removal was reduced in the presence of fructose whose rate of removal increased in turn when glucose had disappeared from the sewage. This demonstration of diauxic growth would be unlikely to be a common phenomenon because of the heterogeneous

state of natural micro-organism assemblages and microhabitat when species succession would actively apply. Certain organic compounds would be processed only slowly or not at all in natural systems indicating the idea of recalcitrance and direct toxicity in substrates (Griffiths, et al 1981; Herbes, 1981).

Micro-organisms will effectively use soluble organic substrates at low natural concentrations (Jorgensen, 1976), but insoluble organic matter makes up a significant proportion of total available organic carbon. Conversion of insoluble to soluble carbon sources is reported to be mediated by enzymes which have been observed in watercourses (Verstraete, et al 1976), lakes (Reichardt et al. 1967), sewage treatment systems, (Sridhar and Pillai, 1974), the sea and marine sediments (Taga and Kobori, 1978; Meyer-Reil, L-A 1981) and will be discussed in detail later.

Of fundamental importance is the level of dissolved oxygen and hence whether the water or sediment presents an aerobic or anaerobic habitat. This will determine the range of flora and fauna likely to colonise the particular habitat. The range of electron acceptors will constrain the conversion of carbon, nitrogen, phosphorus and sulphur in their combined or inorganic state. Whilst bacterial genera will always be present that would be able to exploit a change from aerobic to anaerobic regime, on a macroscopic level such a change would be catastrophic for higher animals such as macroinvertebrates and fish.

1.2 ENZYME ACTIVITY IN AQUATIC ENVIRONMENTS

1.2.1 Introduction

1.2.1.1 Description of Aquatic Environments

Natural environments in which there is an unconstrained aqueous phase, having a budget of matter and energy which then determines the flora and fauna, are common. Aquatic environment therefore is a generalised definition of limited utility because of the extreme diversity of the environments included. These environments are grouped here in order to set a context for a review of the literature which tends to concentrate on certain areas.

Rivers and watercourses in general are the first grouping. Their importance has been pointed out in Chapter 1.1 and their significance from a pollution control viewpoint is continually stressed, as is the potential for turnover of inorganic matter, organic matter and biota. This potential is echoed in the ironic comment by Leopold et al (1964), that rivers are: "gutters down which flow the ruins of continents".

Fluvial systems pose particular problems to organisms attempting to colonise them; deriving from the effects of variable current speed on the need for attachment, the ebb and flow of nutrient concentrations, the periodic resuspension and deposition of bed materials and the dynamic nature of ambient temperature, dissolved oxygen and pH. Drought is an extreme problem, when the habitat may temporarily cease to exist. In the British

Isles such climatic extremes are rare events but in warm climates watercourses with a small groundwater input would tend to be non permanent. At the other extreme are rivers that receive large seasonal inputs of snow meltwater. The effect of such habitat variability on eucaryotic populations including macro- invertebrates and higher plants is a large continuing field of research. Comments on the bacterial flora of rivers have been made in Chapter 1.1.

Lakes and ponds are superficially less dynamic in nature than rivers. Water movements in lakes are not driven principally by gravity with the exception of points of inlet or outlet and seiches, which are oscillations of levels with consequent flow; caused by changes in barometric pressure. Nevertheless there are very significant gross environmental effects in standing bodies of water. These effects include the creation of thermal and chemical gradients both vertically and horizontally. There is also an inertia intrinsic in large masses of water and only a minor side wall effect. Populations of protists, animals and to an extent plants develop within these gradients taking advantage of the possibilities for matter and energy changes. In contrast to rivers, lakes have a large potential for primary production subject to availability of nutrients. Striking demonstration of this productivity is presented by algal blooms in eutrophic lakes. Phytoplankton growth in rivers is often restricted by residence times less than those that are optimum for the generation time of the algae, or low light levels experienced by the cells as the current moves them within the water column when natural turbidity levels are high. Nutrient concentrations in

rivers may be comparable to those in eutrophic lakes where algal blooms are predictable seasonal events.

Marine habitats have been extensively studied. Some properties are found in common with lakes but on a much larger scale and there are environmental extremes of depth, temperature and chemical quality which make the marine environment perhaps the most varied. Estuaries are dynamic habitats which bridge some of the properties of freshwater rivers and the oceans.

Of particular importance to the quality of rivers and lakes are the sites of collection and treatment of waste water. These treatment systems are microcosms of the whole water cycle and the process of self-purification. Sewage in comparison to a natural river or lake is a relatively high substrate medium. Flow regimes are controlled in the process to separate grits and heavy inorganic particles and settleable suspended matter to form sludges which constitute the parallel phase of treatment to the purification of the bulk liquid. Aerobic and anaerobic systems are set up to stabilise and separate pollutants.

Where water is associated with particles in such a proportion that the system is classed as solid or semi-solid a different but very important habitat is created. These high solids environments include soils which clearly have a universal importance, sediments in river and lakes which have been viewed as very wet soils in terms of their chemistry and biology and sludges from sewage works which are high in organic matter and sites of intense biological activity. (Crowther and Harkness, 1975). Within the high solids environment would also be

included permeable substrata with associated groundwater. However water that passes through soil and a zone of permeable unsaturated rock to become groundwater usually has low organic carbon substrate levels and is either sterile or has very low numbers of organisms and biological activity.

The importance of water as continuous phase in a number of environments has been outlined above. In all of these environments enzyme activity has been identified as a biochemical property of ecological significance. Before reviewing this work the major groups of enzyme activities are discussed below and those playing a role in aquatic environments are highlighted.

1.2.1.2 Major Groups of Enzyme Activities

Enzymes generally were classified by the Commission on Enzymes (Florkin and Stotz, 1964) into six general groups dependent on the type of reaction catalysed. These groups are:

1 Oxidoreductases - catalysing oxidation-reducing reactions
2 Transferases - catalyzing group transfer reactions
3 Hydrolases - catalyzing hydrolytic reactions
4 Lyases - catalyze reactions involving the removal of a group leaving a double bond or addition to a double bond.
5 Isomerases - catalyzing isomerisations
6 Ligases or synthetases - catalyze the joining together of two molecules, coupled with the breakdown of the pyrophosphate of ATP.

Major classes are further divided into subclasses, sub-subclasses and finally specific enzymes each with a unique code for identification.

The soil environment is the system upon which more work has been carried out on enzyme activity. Ladd (1978), and Burns (1978) comprehensively reviewed the origin and range of soil enzyme activities, noting that the enzyme groups most frequently studied were oxidoreductases and hydrolases. Other groups investigated included tranferases and lyases but no work had been reported on isomerases and ligases. Most published studies covered research into activities in soil of dehydrogenases, catalase, invertase, protease, phosphatase and urease. Other enzymes investigated include those attacking common storage and structural polysaccharides, such as amylase, cellulase, pectinase and xylanase.

Enzymes in natural waters have been less extensively studied. Studies have often taken as a starting point, published work on soil enzymes so that enzymes measured in waters tend to be in the same groups as soil enzymes. Kakde and Raman (1978), in their review of enzyme activity as an index of activity in waste waters concluded that oxidative and hydrolytic enzymes were the better indicators. They selected the intracellular enzymes dehydrogenase, catalase and reductase as useful markers of energy transactions and protease, urease and phosphatase as markers of breakdown of compounds such as protein urea and phosphoric esters found in sewage.

The measurement of microbial metabolic activity in water samples in terms of eight different enzymes was described by Obst (1985) who recommended the use of chromogenic substrates to assay the activities of esterase (EC 3.2), protease (EC 3.4), α-amylase, (EC 3.2.1), phosphomonoesterase(EC 3.1.3), aminopeptidase, (EC 3.4.1) α-glucosidase (EC 3.2.1.20) and β-glucosidase, (EC 3.2.1.21). The fluorescein diacetate substrate used for this assay is susceptible to hydrolysis by extracellular esterases, proteases and lipases and is thus a broad detector of heterotrophic activity. An electron transport system enzyme NADPH-dependent dehydrogenase (EC 1.1.1) was selected to model the energy producing metabolic reactions.

1.2.1.3 History

Much of the early work on the identification of enzyme activity in aquatic environments resulted from experiments to improve the understanding of sewage treatment processes. Sridhar and Pillai (1966) in their review of enzymes in sewage and sludges cite the work of Thomson (1922) who noted the apparent enzyme catalysed breakdown of maltose, sucrose, proteins and urea. An intimate connection between microbial slimes in treatment filters and the enzyme activities was noted. The pioneering work of Ardern and Lockett (1914) on the development of the activated sludge process incorporated the concept of coagulation of colloidal organic impurities in the sewage by micro-organisms and their extracellular activity. Effective sewage treatment processes were at this time known to depend on oxidation of organic matter by biological means and work by Wooldridge and Standfast, (1936)

established that sewage sterilised by filtration, heat or chemicals would not absorb oxygen. Incomplete inactivation of the bacteria in sewage by for example careful ultraviolet irradiation which killed the bacteria but did not denature the proteins, would still allow uptake of oxygen and partial oxidation of the organic matter.

Work on enzyme activity in soils in the recent past is reviewed by Skujins (1978) who noted that catalase activity was an early subject of study with later work on urease, protease and phosphatase all directed towards a better understanding of soil fertility.

Zobell, (1939) reported work on enzymatically mediated oxygen consumption in marine sediments.

In the more recent past the development of microbial ecology has included important work on the origin and regulation of cell free enzymes such as phosphatases in lakes, their role in eutrophication (Reichardt, et al. 1967); and the contribution of enzyme activity to the cycling of matter in aquatic environments (Fenchel and Jorgensen, 1977).

1.2.2. Occurrence of Hydrolytic Enzyme Activity in Aquatic Environments

1.2.2.1 Rivers and Flowing Waters

Reports of measurements of enzyme activities in watercourses are very few in number. Sridhar and Pillai, (1969) used hydrogen

peroxide as a substrate to measure catalase activity in Indian rivers polluted with sewage. Catalase activity was positively correlated with degree of pollution as expressed as oxidisable organic matter and ammoniacal nitrogen. As would be expected the waters with the worst sewage pollution had the highest bacterial numbers but no attempt was made to count or isolate bacteria showing catalase activity.

A more wide ranging study of phosphatase activity in different aquatic environments including small urban watercourses, a spring, a canal, a lake and sewage treatment plants was reported in 2 papers by Flint and Hopton (1977a, b). Alkaline phosphatase activity associated with particulate matter was measured on both particles and cell free extracts of particles from environments with a range of ambient orthophosphate concentration. It was found that addition of orthophosphate to enzyme assays inhibited the activity of low ambient phosphate extracts more than those from high phosphate environments. Extracts hydrolysed a range of inorganic and organic phosphates but their activity was more inhibited by added anions such as fluoride and molybdate than cations. In their second report they expressed phosphatase activities of water samples, both filtered and unfiltered, in terms of the plate counts of the samples; noting that specific activity had an inverse relationship with orthophosphate concentration of the environment sampled suggesting an induction-repression effect by orthophosphate. The lack of a clear cut picture of the pH profiles of phosphatase activity in the range of sites sampled was attributed to the interplay of different factors such as variable microflora, nutrient and chemical status.

In work aimed at establishing the efficacy of enzyme activity measurements as an index of biological activity in aquatic environments Verstraete et al (1976) assayed the activities of phosphatase (at pH 8.4), saccharase, amylase, lipase, amidase and protease in 2 Belgian lakes (low pollution) and a canal at 3 sites all of which were polluted by sewage or industrial wastes. Of the enzyme activities measured only phosphatase and saccharase were amenable to interpretation, the other activities being too variable.

Both phosphatase and saccharase activities showed strong seasonal fluctuation. Phosphatase was highest in the spring and autumn which supports a link shown with algal activity expressed as chlorophyll content of the sample. Saccharase was at a maximum in the winter months, presumably because of the increased polysaccharide products released from decomposing algal biomass.

Filtration of the samples showed that saccharase was completely associated with suspended solids.

Correlation of saccharase activity with the BOD and bacterial count of the samples at statistically significant levels was confirmed by estimation of enzyme activity and viable count of samples amended with substrates including sucrose, peptone, starch or vegetable oil. Phosphatase activity correlated well with viable count on addition of all substrates but saccharase responded best to sucrose and peptone.

Verstraete et al (1976) contended that enzyme activity methods would be valuable tools for fundamental studies of aquatic environments but that more information on naturally occurring levels would be needed before comprehensive interpretation of the data could be undertaken.

As has been stated, that data base has yet to be built up for most enzymes. Methods are generally not standard. Phosphatase activity using nitrophenyl phosphate as substrate is probably the nearest to a standard technique but final results are expressed in a range of units making comparisons difficult. The apparent lack of published work on enzyme activites in flowing waters may reflect a greater concern currently with the sediment phase of this particular habitat. Whilst it may be that a greater part of the matter and energy exchange takes place in the sediments, the attached vegetation or periphyton in a flowing system, it will not be possible to synthesise an ecological description of the whole system without including the contribution of the aqueous phase.

1.2.2.2 Lakes

Lakes have for a long time been the subject of multi-disciplinary investigation with microbiology being considered an important aspect of any overall view of the ecosystem. (Collins, 1963). Hydrolytic enzyme activity is considered to be an essential feature of two major biological processes of lakes. The first of these processes is the growth of microalgae, their autotrophic production of organic carbon, the role of phosphorus in algal growth and the relationship of

aquatic bacteria to phytoplankton. A second and perhaps even more fundamental process is the breakdown of complex organic compounds, which may be the results of primary or secondary production, and the subsequent cycling and recycling of organic substrates and nutrients.

For lakes receiving minimal input of organic matter from rivers, autochthonous organic matter derives from primary production. The protein content of algal cells varies widely, dependent on species and growth conditions but Golterman (1975) quotes an average figure of 50% of dry weight. Little et al (1979) point out that the rate and extent of solubilisation of this insoluble matter will have a bearing on the degree of anaerobiosis in both the hypolimnion and sediments. They measured proteolysis in water from Lake Champlain using an insoluble azure dye derivative of hide powder. In situ proteolysis was found to be temperature dependent with no measurable activity below 4°C. Bacteria were allowed to grow in the reaction vessels and these were predominantly Pseudomonas and Flavobacterium species; in addition it was noted that filter sterilised lake water did not show activity. It is therefore difficult to extrapolate results from these proteolysis flasks to the conditions naturally occurring in the lake.

The enzymatic breakdown of particulate and dissolved proteinaceous matter in a eutrophic lake was investigated by Halemejko and Chrost (1986) using hide powder azure as an insoluble substrate and synthetic peptides as dissolved substrates. They found that significant proteolytic activities were observed with higher rates in the epilimnion in comparison

to the profundal zone. There was also a peak of proteolysis and aminopeptidase activity following the breakdown of spring and summer phytoplankton blooms. An even closer link with algal activity was shown by the diurnal variation of both aminopeptidase and endopeptidase activities.

Whilst no bacteria counts were performed by Halemejko and Chrost (1986), the work of Chrost et al (1986) on the distribution and numbers of heterotrophic bacteria and the occurrence of protease, amylase, lipase and phosphatase producing micro-organisms in the same eutrophic Polish lake complemented the previous work by demonstrating that maxima of bacterioplankton occurred after the breakdown of phytoplankton blooms. Proteolytic bacteria were the major group of heterotrophic bacteria counted; but the authors point out that there may be a discrepancy between counts on relatively rich media used for plate counts and metabolic tests, which would favour saprophytic bacteria that can tolerate the high levels of organic matter, in comparison to natural lake water counts where the so called oligotrophic bacteria (Kuznetsov et al. 1979) which normally have high substrate affinities, would have difficulty adapting to the rich media.

The origin, nature and regulation of phosphatase activity in lakes have been extensively covered in reviews and reports (Reichardt et al. 1967; Berman, 1970; Jones, 1972; Pettersson, 1980; Currie et al 1986; Flint and Matavulj, 1987).

The factors controlling algal growth in lakes have been studied and phosphorus limitation is often noted (Berman, 1970). A mechanism whereby algae could overcome this shortfall was demonstrated by Heath and Cooke (1975) in a eutrophic lake where a blooming blue green alga Aphanizomenon flos-aquae produced a phosphatase activity which could hydrolyse phosphomonesters that were also detected under conditions of low orthophosphate concentration. Berman (1970) measured alkaline phosphatase activity and total phosphorus concentrations in Lake Tiberias noting an effect analogous to the classical induction-repression mechanism. Partitioning of phosphatase activity between particulates and water had been shown by Reichardt et al (1967) in the Pluss-See where about 90% of activity was retained by a 0.6 μm filter. They also found a correlation between concentration of free enzymes in the lake water and the numbers of bacteria and phytoplankton.

A more recent study comparing the uptake of orthophosphate and association of alkaline phosphatase activity with algal and bacterial elements of the microflora of lakes of differing trophic status reported that phosphate uptake was largely associated with particles of a sub algal size but that phosphatase activity was either free in solution or associated with algae. (Currie et al. 1986). This finding challenges the common theory that algae are the main users of orthophosphate in such environments. A clear picture of the relationship between algal and bacterial phosphatases and the role of inorganic and complex phosphates as nutrients for these organisms has yet to emerge.

Phosphatase enzymes are not found exclusively in algae and bacteria but are produced and utilised by other aquatic organisms. Daphnia magna cultured in the laboratory was found to release a soluble alkaline phosphatase and small quantities of acid phosphatase. (Boavida and Heath, 1984).

1.2.2.3 Marine Environments

The occurrence and activities of cell free enzymes assayed in the oceans have been reviewed by Kim and Zobell (1974) who detected phosphatase, amylase, urease, protease and dehydrogenase activity in significant amounts in water and sediment samples from the Pacific Ocean and saline lakes.

Earlier work had shown the common occurrence in marine sampling sites of bacteria producing proteolytic activity (Merkel et al 1964). Prescott and Wilms (1960) had shown that proteolytic activity could be extracted from cultures of bacteria isolated from the sea and grown on synthetic media. Activity levels of the extracts against a haemoglobin substrate were high. Film forming periphytic marine bacteria of the genus Pseudomonas isolated by Corpe and Winters (1972) were grown in bulk in a glucose peptone medium. Hydrolytic enzyme activity was detected free in the culture medium, in cell extracts or bound to cell envelopes. The greatest amounts of proteinase, esterase, phosphatase and β -glucosidase were located in the cells. All the bacteria tested showed some enzyme activity against a range of algal and bacterial polysaccharides.

More recently Vives-Rego et al (1986) used the L-leucyl-napthtylamide method of Somville and Billen (1983) to investigate the inhibitory effect of heavy metals and surfactants on exoproteolytic activity in sea water from the Mediterranean Sea. They reported an inhibitory effect of lead, nickel, chromium and zinc ions but an observation that cadmium ions had no effect below 50 mg/l. Surfactants appeared to enhance proteolytic activity which is difficult to explain unless activity was being released from particles by the surface activity of the chemicals.

Methylumbelliferyl substrates were used by Hoppe, (1983) and Somville, (1984) to investigate the exoenzymatic hydrolysis of α and β -glucosides, protein-like compounds, glucosaminides and organic phosphates, in eutrophic Baltic fjords and the North Sea. Hoppe (1983) found that protease activity was dominant in all samples, that α-glucosidase and glucosaminidase varied widely between the brackish fjords and the offshore waters. He concluded that enzyme activity was a useful indicator of organic pollution and eutrophication. Somville (1984) found that both α and β -glucosidase activity could be readily measured using the fluorogenic substrates; was associated with particles larger than 0.2 μm and was linked to bacterial activity determined by thymidine incorporation.

1.2.2.4 Enzyme Activity in Waste Treatment Systems

The presence of enzyme activity in sewage and organic waste water treatment systems has been extensively reviewed (Siddiqi et al, 1966; Sridhar and Pillai, 1966; Carroad and Wilke, 1978;

Kakde and Raman, 1978 and Stavskaya and Volchenkova, 1979). Published studies tend to concentrate on the hydrolytic enzymes as indicators of degradative powers of treatment biomass or the efficiency and possible degree of inhibition of biomass as assessed by the activity of oxidoreductive enzymes such as dehydrogenase.

There is an important economic dimension to the process of waste treatment and there is always concern to optimise the efficiency of systems whether in terms of energy consumption to maintain aerobic systems or in terms of retention times in such treatment units as anaerobic digesters or settlement tanks where reductions would allow large savings on civil engineering and operational costs. The exploitation of enzyme activities in these systems is part of the constant search for greater efficiency. More recently the interest in conversion of wastes to useful by-products in industries with low volumes of strong effluents and thus high substrate concentrations, as compared to the high volume low substrate concentrations in crude sewage, has focussed on the role of enzymes either free or associated with micro-organisms. (Dunnill and Rudd, 1984).

The bacteria bed or biological filter method of secondary aerobic method of treating sewage has been studied in kinetic terms to produce predictive methods of BOD removal (James, 1984) or more specifically to describe the mechanism of transport of various substrates into the thick biofilms attached to the filter media (Taylor-Eighmy and Bishop, 1984). Little research appears to have been centred on the occurrence and action of enzymes in these microbial films.

In contrast the range of enzyme activities detected in the activated sludge system is wide. Teuber and Brodisch, (1977) were able to detect measurable activity of phosphomonoesterase, cyclic phosphodiesterase, glycosidase and aminopeptidase within 10 to 20 minutes incubation of nitrophenyl or nitroanilide substrates with diluted activated sludge at 30°C. 95% of the activity sedimented together with the flocs. A comparative study of the removal rates of chemical oxygen demand for a series of 13 different activated sludge plants treating a wide range of sewages was carried out by Richards et al (1984) who also measured the activity of a range of hydrolytic enzymes and a dehydrogenase. They concluded that α-glucosidase, L-alanine aminopeptidase and protease had potential for use as simple assessors of overall sludge activity.

Hankin and Sands (1974) used a range of solid culture media formulated to be selective for heterotrophic bacteria degrading specific substrates and to count the numbers of such bacteria in the stages of treatment of several activated sludge plants. They counted significant numbers of bacteria capable of degrading detergents, cellulose, DNA, RNA, pectin, lecithin, protein, starch and lipid at all stages of treatment but noted large reductions in some types such as amylolytic bacteria between treatment stages.

Two enzymes which were the subject of early studies of enzyme activity in sewage and more recently in activated sludge systems were catalase (Vaicum et al 1965) and urease (Lenhard, 1969; Sridhar and Pillai, 1974).

Sludges produced during sewage treatment have high organic matter content and anaerobic conditions are rapidly established. Treatment under anaerobic conditions is carried out to stabilise the sludges to render them amenable to dewatering or to be less hazardous if disposed of to land. Hydrolytic enzymes play a very important role in the breakdown of polymers such as starch, cellulose, protein and particularly lipids prior to absorption and fermentative breakdown of the hydrolysis products by the indigenous bacteria. Thiel and Hattingh (1967) prepared cell free extracts of anaerobic digesting sludge and described the measurement of amylase, protease, cellobiase and phosphatase activity. Acid and alkaline phosphatase activity was found by Ashley and Hurst (1981) to be a valuable indicator of overloading of sludge digesters giving 10 days prior warning of the appearance of higher molecular weight fatty acids which are the conventional sympton of digester failure.

Industrial waste treatment systems have been less well studied than those receiving domestic or mixed sewage and may well have restricted microflora because of the nature of the waste. A fellmongery waste containing sulphides, mucopolysaccharides, high calcium and ammonium concentrations, emulsified fats and the wool fibres associated with dehairing sheepskins was treated by Rawlings and Woods (1978) in a laboratory activated sludge plant. They noted that protease, catalase and phosphatase activity increased rapidly but that the microflora was dominated by proteolytic and lipolytic bacteria with few that could degrade starch, cellulose or detergent.

1.2.2.5 Enzyme Activity in Sediments and Soils

Particulate organic carbon and nitrogen compounds in rivers, lakes or marine environments whether derived autochthonously or from the catchment, eventually settle out of the water column to form sediments. On land, the analogous process of litter production results in an incorporation of carbon and nitrogen containing detritus into the soil.

In sediments, the organic carbon provides a source of reducing power which drives the geochemical cycles. This complex system of processes was reviewed by Jones (1985) in lake sediments where polymer hydrolysis and monomer decomposition, linked to the activity of heterotrophic bacteria, are early stages in the process.

Although the range of polymeric organic materials in sediments would be expected to be wide the number of hydrolytic enzyme activities detected in freshwater sediments from watercourses or lakes is restricted. This may be because only surveys with limited scope have been undertaken. A study by Duddridge and Wainwright (1982) found that the activity of amylase, cellulase and urease in natural sediments from four clean rivers were very low until amended by the addition of substrates for those enzymes. They then found that activities could be modelled by Michaelis-Menten kinetics and were inhibited to a marked degree by heavy metals (Wainwright and Duddridge, 1982).

Jones (1979) took amylase and protease activities as indicators of hydrolytic action in lake sediments in relation to changes in redox conditions. The enzyme activity was found to be highest at the sediment surface and decreased steadily with depth into the sediment in contrast to the electrode potential which showed a rapid fall. Electrode potential gradients are sites of high metabolic activity (Fenchel and Jorgensen, 1977) and it might be expected that enzyme activity would be high or variable in these environments.

Phosphatase activity has been studied in freshwater sediments from a range of lakes and streams by Sayler et al. (1979) who found that alkaline phosphatase activity correlated well with measures of bacterial biomass (ATP) and viable counts of bacteria. Baker and Morita (1983) used neutral phosphatase activity and other metabolic measures such as nitrogen fixation and glucose uptake rate to measure the effect of crude oil added to sediments from clean streams noting that as little as 0.1% oil concentration, which nevertheless is 1000 mg/l, reduces the rate of glucose uptake and enzyme activity significantly.

A much broader spectrum of hydrolytic enzyme activities have been measured in marine sediments. Proteolytic enzymes, α-amylase and β-glucosidase were measured by Meyer-Reil, (1981) in the brackish water sediments of the Kiel Bight. Activities were strongly dependent on temperature but with significant activity at 5°C and a greatest increase as the temperature increased from 15 to 20°C. Lower salinities of 0 to 0.8‰

favoured enzyme activity. All three activities were closely linked through concentrations of proteins and carbohydrates with seasonal breakdown in algal blooms.

In addition to protease and amylase Griffiths et al (1983) measured cellulase, laminarinase and xylanase activities in Bering Sea sediments. In a detailed study of these hydrolytic enzymes and a range of microbial metabolic variables they found significant concentrations of enzyme activities and attempted to explain these activities in terms of established measures of microbial activity such as glutamate uptake and glucose uptake and respiration. They found a complex pattern of inter-relatedness of factors and concluded that enzyme activity "probably reflected relative levels of microbial heterotrophic activity".

In an earlier study on sub-arctic marine sediments, amended with storage and structural polysaccharides and treated with crude oil, Griffiths et al. (1982) found that the activity of enzymes attacking structural polysaccharides: cellulose, chitin and laminarin were decreased and those attacking storage polysaccharides: starch and alginate were increased by the presence of crude oil.

An in situ study of cellulose decomposition and the effect of different zinc concentrations in a sediment from Dublin Bay carried out by Picaver and Lyes (1981) showed that whilst in regions of coastal water organically polluted by sewage, which also caused the high metal concentrations, the metabolic

activity and cellulose decomposition was higher than in the clean water, the relative rate expressed per unit bacterial numbers was five times lower in the polluted site.

Little if any uniformity in assay conditions or conventions for reporting activities exists at present with the result that comparisons of findings between different activities and different sediments, tend to be qualitative rather than quantitative. Sediments also vary widely in their mineralogy, content of organic compounds, nutrients and in the natural state their flora and fauna. All this adding to the problems of interpretation of observation.

Knowledge of the structure and chemistry of soils is much more extensive however and soil enzyme activity has been studied at many sites around the world. Reports of data on soil enzymes have been comprehensively reviewed by Burns, (1978). Hydrolytic enzymes have been particular subjects of study because of their putative role in cycling of organic matter in the soil. A much wider range of polysaccharidases have been measured in soils (Kiss, et al 1978) then in sediments.

Despite the wide range of enzymes reported there is no clear theory current as to which activities have dominant roles. Enzymes such as phosphatase would play an important role in phosphorus cycling, (Verstraete and Voets, 1977), protease is central to turnover of nitrogen (Ladd and Butler, 1972), cellulase and amylase act on very common polysaccharides that need to be broken down (Jones and Grainger, 1983) and urease

would be important where the soil received treatment with animal wastes (Nannipieri et al 1986; Ross, et al. 1982).

The existence of many reports on soil enzymes is used to extrapolate findings into studies of enzyme activities in aquatic environments. Similar methods are also used. This is a legitimate practice because of the environmental continuity between soils, groundwaters, watercourses and lakes.

1.2.3 Nature and Role of Enzyme Activity in Aquatic Environments

1.2.3.1 Location of Enzyme Activity

Enzyme activity has been reported in a range of aquatic environment with some authors attempting to localise the activity. Reichardt et al. (1967) detected and investigated the properties of dissolved alkaline phosphomonoesterase activity in lake water passed through a 0.6 micron membrane filter although this free activity was a small proportion of that in unfiltered water. Cell free enzymes, amylase, alkaline phosphatase, urease and protease were recovered from seawater sediments sampled by Kim and Zobell (1974) off the California coast.

Whilst cell free enzyme activity can be demonstrated, a greater proportion of activity resides in suspensions of particles either dilute as in lake water or much more concentrated as in sediments or sludges. Teuber and Brodisch (1977) found that the average activity in the particles separated from an activated sludge by centrifugation for 6 peptidases, 4 glycosidases and 2 phosphatases was 85% of the total activity.

It may be that some non-living particulate matter has a catalytic activity on substrates such as proteins or polysaccharides but it has been demonstrated by Somville and Billen (1983) that autoclaving of samples of seawater causes a complete loss of exoproteolytic activity.

The association of hydrolytic enzymes with bacteria was investigated by Corpe and Winter (1972) in marine Pseudomonads grown on a complex nutrient medium. Cell suspensions were harvested by centrifugation with extracellular enzymes being measured on the supernatant. Washed cells were either suspended in sterile seawater with toluene as bacteriostat and shaken at room temperature to allow autolysis of cells, or sonicated to produce cell envelopes and cell contents then termed an extract. Extracellular proteinase and esterase but no phosphatase or amylase was found. Most activity was associated with cell envelopes (cell membrane and cell wall) with some activity such as proteinases being relatively easily washed off the envelopes probably from the periplasmic spaces. Autolysis also resulted in loss of activity from the cells to the medium.

In sediments and particularly soils, the microbial activity is intimately connected with and determined by the nature of the soil particles. Burns (1978) reviewed the interactions of enzymes and the soil environment concluding that the clays and organic colloids with their high unit surface area were very important in the microbial ecology. Many mechanisms existed to cause adherence of microbes, enzymes substrates and products to soil surfaces including: ion exchange, covalent bonding, hydrogen bonding, secretion of exopolysaccharides, physical

attachment by pili and lipophilic association with organic matter. Complex pH and inorganic nutrient gradients could exist at interfaces between particles and water resulting in effects on enzyme activity in the so called diffuse double layer. Association of microbes and particularly enzymes in clay-organic colloid-humus aggregates results in a general attenuation but stabilisation of activity.

It would not normally be ecologically advantageous for an organism to secrete potential substrates, which is what extra cellular enzymes would be, unless the products of their activity were readily available for absorption. This situation would pertain if substrates moved into the cell envelope where enzymes were located or where there was a very stable and intimate association of enzyme, substrates and cells. This appears to be the case in the soil-aggregate microenvironment.

No reports of comparable work on the microenvironment of the high water content aquatic sediments or seston ic particulates have been found.

1.2.3.2 Reaction Kinetics of Enzyme Activity

Many researchers having detected enzyme activity in aquatic environments or isolated organisms then test the activity to confirm its enzymatic nature. Criteria set out by Kim and Zobell (1974) include the protein nature of the catalyst which will have a pH and temperature profile of activity including denaturation at extreme values and a specificity of action.

Verstraete et al. (1976) in standardising assays for phosphatase, amylase, saccharase and lipase on polysaprobic canal waters carried out experiments to test whether increase in product formation was linear with time of incubation and sample concentration. Optimum pH conditions for activity were different for each enzyme with phosphatase showing peaks in both acid (pH 5.5) and alkaline (pH 8.4) conditions. pH and temperature profiles will tend to be variable and not necessarily clear cut because of the possibility of multi-enzyme systems being found in different aquatic environments. Reichardt et al (1967) found three peaks of phosphomonoesterase activity between pH 4 and pH 10. Duddridge and Wainwright (1982) found a sharp peak of activity of amylase activity at 45°C falling to less than 3% of the maximum at 75°C in 4 different river sediments. Reichardt et al (1967) quantified the dependence of alkaline phosphatase activity in lake water on temperature between 5°C and 35°C by presenting activity data as an Arrhenius plot. This plot may give some insight into enzyme activities originating from organisms adapted to different temperature regimes.

The more detailed relationship of rate of enzyme catalysed reactions and substrate concentrations is investigated by fitting kinetic models to the data from assays. A Michaelis-Menten or saturation kinetics model is most frequently fitted in which the initial rate of reaction (v) is expressed as a function of the initial substrate concentration (s) by the

Michaelis-Menten equation:

$$v = \frac{V \cdot s}{(Km + s)}$$

Where V is the maximum velocity and Km is known as the Michaelis constant. The Michaelis constant Km is a characteristic constant for each enzyme having the dimensions of concentration. It is equal to the concentration of substrate when the reaction proceeds at half the maximum velocity and represents the reciprocal of the affinity of the enzyme for its substrate. There is no uniformity as yet in the assay conditions or units reported but estimates of Km for phosphatase activity are more amenable to comparison because the substrate nitrophenyl phosphate is commonly used. Reichardt et al (1967) quote a figure of 1.1×10^{-6} molar which compares with a range of 0.1 to 5×10^{-6} molar by Pettersson (1980) on lake water and sediments. In contrast a general figure quoted by Spier and Ross (1978) supported by work carried out by Pettit et al (1977) for soil phosphatase was 350×10^{-6} molar. The difference might reflect the constraints on substrate movements in the high solids environment of soils. Comparison for other activities is difficult but Halemejko and Chrost (1986) using dye-substituted peptides and Jackman et al (1983) investigating the action of proteases purified from a psychotrophic Pseudomonad on a casein substrate obtained estimates of Km of 35 and 40×10^{-6} Molar. A figure quoted by Sridhar and Pillai, (1986), for protease extract from activated sludge with casein as substrate was 34000 mg/l which is about ten times the figures quoted above, illustrating the wide range of activities.

1.2.3.3 Regulation of Enzyme Activity

A knowledge of the factors, whether environmental or biotic, which control the production and action of enzymes is an aim of research on the role of enzyme activity in aquatic environments.

Regulation of the control of enzyme production was the subject of the classical work by Jacob and Monod (1961) and their model of the genetic regulatory mechanisms in the synthesis of proteins has been used by workers in the field of aquatic microbial ecology.

Enzymes which attack exogeneous substrates, such as the B-galactosidase system studied by Jacob and Monod (1961), are generally inducible. Duddridge and Wainwright (1981) reported a substantial increase in activities of amylase and cellulase in river sediments to which the respective substrates were added. An increase in numbers of substrate hydrolysing bacteria also occurred. These authors chose the term stimulation of enzyme activity rather than enzyme induction which implies a much more specific process involving the interaction of a low molecular weight intermediate with the repressing system within the cell. Verstraete et al (1976) however do refer to the induction of phosphatase and saccharase activity of natural water samples by the addition of peptone, sucrose, starch or vegetable oil. They report that phosphatase is increased in all cases whereas saccharase is increased in only three of the four cases indicating that activity can be stimulated by a range of organic substrates. Studies of enzyme induction can only be properly

carried out on specific organisms in culture. A study by Daatselaar and Harder (1974) on a marine bacterium showed that proteolytic enzymes were only produced in response to the presence of a narrow range of specific amino acids.

Induction of α and β -glucosidase was reported by Somville (1984) in samples of pond water supplemented with the x glucosides maltose or starch or the β glucoside cellobiose. Comparison with samples supplemented with glucose showed a change in the ratio of α to β -glucosidase dependent upon which glucoside was present as a supplement.

Induction of extracellular enzymes in the presence of exogeneous substrates may be the result of hydrolysis of the substrate by low basal activity of a constitutive enzyme producing low molecular weight products that enter the cell inducing further enzyme production.

The repression of enzyme activity in aquatic environments has been studied most commonly in the phosphatase enzymes. Berman (1970) and Kobayashi et al (1984) have demonstrated an inverse relationship between enzyme activity per unit biomass (or bacterial cell) and inorganic phosphate ion concentration in lake waters. This relationship is characteristic of derepressible enzymes. There is also evidence of inducible alkaline phosphatase activity put forward by Heath and Cooke (1975) who found an inverse relationship of activity with phosphomonesters. The presence of phosphomonoesters induced a production of enzyme activity which in turn caused a reduction in organic phosphate concentration. The overall regulation of

phosphatases in natural environments compared with in vitro systems is far from clear as evidenced by Matavulj and Flint (1987) who in formulating multi-element regression models of acid and alkaline phosphatase activities in a small pond pointed out the many inter-related physical, chemical and biotic factors such as the variable contribution of bacterial or algal biomass, which had to be taken into account.

Enzymes once produced and in place in the cell or in one of the many extracellular locations are subject to a wide range of inhibitory factors and mechanisms which regulate their catalytic action.

Hoppe (1983) and Somville (1984) used transformations of the Michaelis-Menten equation to plot data from assays of peptidase and glucosidase activity in marine samples using substrates and substrate homologues. The relationship of Km and Vmax for different substrates indicates the nature of inhibition and the likelihood of naturally occuring substrates being responsible for networks of mutual regulation. The use of Michaelis-Menten kinetic models to assess chemical toxicity in aquatic environments has been reviewed by Bitton (1983). Kinetic studies by Katayama-Hirayama (1986) quantified the different toxicities of heavy metals towards the β-galactosidase and dehydrogenase activities of activated sludge.

1.2.3.4 Role of Enzyme Activity in Processing of Organic Matter

The role of certain enzymes such as catalase and urease which have simple substrates is probably straightforward. Phosphatase

enzymes would seem to be produced in order to make phosphate available for both heterotrophic bacteria and autotrophic organisms such as algae, but as pointed out by Currie et al (1986) the partitioning of phosphorus between these organisms is far from simple.

For the hydrolases that breakdown proteinaceous matter, structural polysaccharides such as cellulose chitin and lignin, or storage polysaccharides such as starch, the role and mode of action of enzymes is complex.

A link between extracellular enzyme activity and uptake of breakdown products has been shown for exproteolytic activity and amino acid uptake by Somville and Billen (1983), Griffiths et al (1983). Work by Hoppe (1983) on peptidase activity and uptake of labelled leucine suggest that a significant proportion of the leucine released from the peptide would not be taken up and would be available to go into the pool of dissolved organic matter.

Chrost et al (1986) point out that dissolved organic matter pools are kept at low levels by rapid turnover of labile species mediated by microheterotrophs. Particulate organic matter however which has high proportions of protein particularly after algal blooms is the the natural substrate for extracellular hydrolytic activity.

Extracellular and particle associated activity is the result of a relatively slow build up of activity originating from live

cells, dead cells and cell fragments; stabilised by organic colloids and mineral particles (Sizemore et al 1973; Corpe and Winter 1982) and able to associate in equilibrium with particulate organic matter.

Cycling of matter extends upwards in food chains as bacteria are a food source for higher organisms such as protozoa (Zobell and Feltham, 1938) which linked with seasonal effects and variable inputs of both potential substrates and potential producers of activity combine to create a complex budget of material and energy.

1.2.4 Assay Methods

1.2.4.1 Introduction

Oxidoreductases and hydrolases are the major groups of enzymes currently of interest in soils and natural waters and have assay methods different in principle. Within each group there are many versions of basic methods.

A typical method of measuring dehydrogenases activity, or as expressed in metabolic terms the electron transport system (ETS) activity, was described by Reichardt (1979) and involved the incubation of samples of seston and sediment from Lake Constance, with the electron accepting redox dye triphenyl tetrazolium chloride. Reduction of the dye produced the coloured triphenylformazan which was measured spectrophotometrically. No natural substrate is added in this method in contrast to assays of malate dehydrogenase and lactate

dehydrogenease carried out by Jones (1979) on sediments from Lake Windermere when oxaloacetate and pyruvate were reduced by NADH; the decrease in fluorescence of NADH in the assay mixture being the measure of activity. Dehydrogenase activity is intracellular, usually membrane bound and its assay thus involves an extraction stage. The use of agents such as chloroform or toluene to inhibit microbial action (Ross, 1968) destroys ETS activity and assays have to be of short duration to avoid the effects of microbial growth and proliferation.

Hydrolytic enzyme activity results in the production of soluble fragments of larger often polymeric compounds. More common polymeric substances include proteins, lipids, nucleic acids, a wide range of polysaccharides such as cellulose, starch, acid mucopolysaccharides, and glycoproteins and condensed phosphates or phosphate esters of sugars, lipids or amino acids. Enzyme activity can be measured by any method that reproducibly analyses the breakdown products of the polymers.

1.2.4.2 Enzyme Activities Chosen for the Study

Two enzyme activities were selected for this study. These activities are the result of the action of numerous enzymes produced by the microflora rather than a single enzyme. The enzymes may be truly extracellular and in solution, bound to particulate matter or associated with the biomass.

Both of the enzymes selected are in the Class 3 of the scheme proposed by the Commission on Enzymes 1961 the details of which are described by Florkin and Stotz (1964). Class 3 are the

Hydrolases or hydrolytic enzymes. The importance of the hydrolytic activities of aquatic bacteria in the cycling of organic matter to release organic carbon and nutrients such as nitrogen, phosphorus and sulphur was recognised at an early date (Waksman et al 1933) and has been reiterated and reviewed frequently in the intervening years (Corpe and Winter, 1972; Kim and Zobell, 1974; Meyer-Reil, 1981; Halemejko and Chrost, 1986).

This study was concerned with the role of heterotrophic bacteria in self-purification of organically polluted aquatic environments and particularly the hydrolytic enzyme activities of these organisms.

Protease was chosen as a suitable indicator for the breakdown of proteins and organic nitrogenous matter containing peptide bonds susceptible to this enzyme. Proteolysis; the solubilisation of proteins, is essential to produce low molecular weight compounds such as amino acids which can be utilised by micro-organisms for metabolic purposes. Billen, (1982) reports that organic nitrogen derived from phytoplankton production or by decomposition, in natural waters consists of about 85% proteins and peptides with only small amounts of free amino acids. Proteolytic organisms are commonly isolated from natural waters where they can form a major part of the heterotrophic flora. (Little et al, 1979). Measurement of proteolysis in natural waters could provide information with which to explain the role of proteolytic bacteria.

Another major group of organic compounds present in natural waters are the polysaccharides and they are stated by Kiss et al (1978) to be the most abundant organic compounds in nature. Many enzymes systems are involved in the stepwise breakdown of the very wide range of polysaccharides and several of these have been studied in natural waters. Polysaccharides tend to have either a structural role (for example cellulose) or a storage role (for example starch). A disaccharide having a storage role is sucrose and the enzyme breaking sucrose down, saccharase, has been extensively studied in soils but much less studied in natural waters. There seems no obvious reason for this apart from the likelihood that sucrose would be expected to be present in very low background levels in natural waters as opposed to other disaccharides such as the α-glucoside maltose and β-glucoside cellobiose which are breakdown products of the common polysaccharides starch and cellulose respectively. α and β-glucosidases are more commonly measured in natural waters (Somville, 1984).

Saccharase was chosen as the second enzyme to be measured bearing in mind that low natural levels of substrate would probably lead to low levels of activity. Also the apparent lack of published references to surveys of saccharase in natural waters would encourage efforts to discover what actual levels of activity are in such waters.

1.2.4.3 Assay Methods for Protease

Protease has the alternative trivial named of proteinase or peptidase but the EC number for this enzyme cannot be specified

because the activity measured cannot be said to be related to a single catalysed reaction. Pepsin or gelatinase has the EC number 3.4.4.1. being a peptide peptidohydrolase which hydrolyses peptides including those with bonds adjacent to aromatic or dicarboxylic L-amino acid residues.

Methods for measuring proteolytic activity revolve round the type of substrate used. Proteins are large complex molecules whose solubility in aqueous solutions is subject to subtle changes in pH, ionic content and temperature. Studies of proteolysis in natural waters generally relate to the turnover of insoluble or particulate proteinaceous material and the substrates chosen reflect that interest.

Common features of the methods are: incubation temperatures of 25 to 40°C, pH of reaction in the range 7.5 to 9.5. Times of incubation vary from less than 1 hour to a period of days depending on the relative activity of the samples and the sensitivity of the method for measuring the reaction products. For longer incubation times a bacteriostatic or bactericidal agent such as toluene or chloroform is added to the reaction mixture. At the end of the incubation time the reaction is terminated by a variety of means dependent on the substrate used.

Casein is a commonly employed substrate, either in a form modified only to improve its solubility characteristics or in a form combined with a coloured moiety; usually an azo dye (Corps and Winters, 1972). Proteolytic enzyme attack on soluble casein

releases peptides of varying length and amino acids in a concentration dependant on the activity level of the enzyme concerned. The termination of the reaction by the addition of an agent such as trichloroacetic acid, TCA, (Jackman et al. 1983) which precipitates unaltered protein but leaves a solution in TCA of the reaction products allows the assays of these reaction products. Lead salts (Batistic et al. 1980) or perchloric acid (Thiel and Hattingh, 1967) have been used to stop reactions in preference to TCA. Reaction products are assayed as amino acids or amino acid derivatives by reagents such as ninhydrin (Sissmore et al. 1973) or for the amino acids containing aromatic rings tyrosine, tryptophan and phenylalanine by measuring absorption of ultraviolet light between 260 and 290 nm (Merkel et al. 1964). Tyrosine has a phenolic group which is detected by the production of a dark blue complex on reaction with Folin-Ciocalteau reagent. (Lowry et al 1951; Sridhar and Pillai, 1973).

Azocasein substrates release a blue coloured azo dye from the protein-dye complex and this is usually measured at 520 nm. A substrate which is particulate is Hide Powder Azure HPA which releases a blue dye on breakdown. This dye is measured spectrophotometrically at 595 nm. (Little et al 1979).

1.2.4.4 Assay Methods for Saccharase

Saccharase has the EC number 3.2.1.26 with recommended name β-fructofuranosidase; systematic name β-D-fructofuranoside fructohydrolase and trivial names including invertase, sucrase, saccharase and β-fructosidase. As has been outlined there have

been a limited number of reports of measurements of saccharase in natural waters (Verstraete et al 1976) but this enzyme has been studied widely in soils as reviewed by Ladd in Burns (1978).

Several authors including Verstraete et al (1976) measure the concentration of reducing sugars, glucose and fructose, resulting from the action of saccharase on the sucrose substrate (Batistic et al 1980; Ross, 1983). Amylase is a polysaccharide hydrolase that produces glucose subunits as end products of breakdown of starch and these can be measured as reducing sugar (Thiel and Hattingh, 1967). Other related α and β-glucosidases have been studied using methylumbelliferyl substrates (Hoppe, 1983; Somville, 1984).

For most enzyme activities studied in aquatic environments there is a problem with non standardisation of assay conditions and lack of uniformity in activity units reported. This makes a comparison of data from different reports very difficult.

1.3 AIMS OF THE INVESTIGATION

Enzyme activities (this term includes intracellular bacterial enzymes, extracellular enzymes and particle associated enzymes) have been measured in a wide range of environments all of which have as an essential feature an unconstrained aqueous phase. This aqueous phase is open to environmental influence and will have a material and energy budget set by the environmental factors and not generally under the homeostatic control of organisms apart from on a micro-scale. These environments range from oligotrophic oceans, lakes and freshwater streams through

sewage treatment systems, polluted rivers and estuaries, organic sludges and sediments to soils. Enzymes studied also cover a metabolic range tending to concentrate on oxidation-reduction systems such as dehydrogenases, or hydrolytic systems such as phosphomonoesterases. An objective has been to extend the explanation of the role of the bacteria in the ecology of the environments in terms of the changes in the natural concentrations of substrates and redox conditions brought about by enzyme activity or the effects of toxic chemicals on the organisms and to a lesser extent on the enzymes.

Self-purification brings about the return or approach of a polluted system to a natural state. This process has implications for the users of the aquatic environments and is of concern to the riparian owners and water undertakings such as Regional Water Authorities. Most established work on self purification has been focused on levels of degradable organic matter and associated dissolved oxygen levels. More recent work investigates heterotrophic activity including enzyme activity of the micro-organisms in the system. Consequently any work that will increase understanding of the metabolic processes going on in aquatic environments also helps to explain the overall complexity of self-purification.

This study had an overall objective of starting investigation of the role of enzyme activity in watercourses and testing any conclusions from other reported work in a local context. The aims of the study are listed below.

Aims

1 To test the applicability of two published enzyme assay methods; one for saccharolytic and one proteolytic activity in local freshwater rivers. Development work was to be carried out to adapt the methods to local conditions.

2 To carry out a preliminary survey of enzyme activity in selected natural freshwaters including treated waste waters to confirm or otherwise that enzyme activity is a common feature of natural waters, particularly those with moderate to severe organic pollution. A range of conventional chemical determinands including biochemical oxygen demand, suspended solids, ammonia, oxidised nitrogen, pH and electrical conductivity were to be measured for comparison. Plate counts were to be carried out on the samples and field determinations on site were to be made on the temperature, dissolved oxygen and discharge of the waters.

3 To investigate the statistical distribution of natural variability of enzyme activity in the natural waters sampled and test models of distributions against the sample data in order to ensure that appropriate statistical procedures were used in processing environmental data.

4 To test a working hypothesis that enzyme activity was a measure of organic pollution by comparing enzyme activity data with data on conventional measures of organic pollution

such as biochemical oxygen demand, physical characteristics such as temperature and discharge and numbers of heterotrophic bacteria expressed as plate counts. Enzyme activity may have potential use by the Water Authority as an indicator of pollution in natural waters.

5 To formulate a regression model of enzyme activity in terms of the characteristics listed in 4 above and test the power of this model to predict enzyme activity in different waters.

6 To isolate bacteria from natural waters and subject them to identification procedures to establish their identify to the genus level. Changes in numbers or types of bacteria in waters may reflect the environmental stress.

7 To culture the isolated bacteria under different nutritional conditions to determine the effect of culture conditions on the production of enzyme activity.

8 To grow selected organisms, identified as producing enzyme activity, under specified conditions with subsequent production of crude enzyme extracts.

9 To carry out preliminary investigation of the nature and kinetic properties of the enzymes in crude extracts and allow comparison with similar published work.

2 MATERIALS AND METHODS

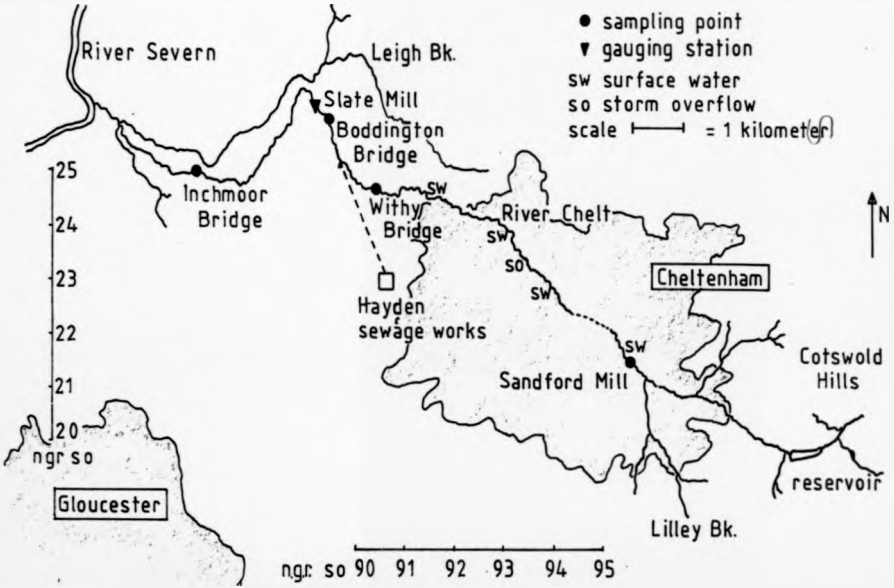
2 MATERIALS AND METHODS

2.1 SYSTEM STUDIED

The system chosen for the initial survey is a small lowland river, the River Chelt, which during its progress from source to confluence undergoes changes in physical, chemical and biological character including a major and sustained polluting input. Fig 2.1 is a schematic drawing of the river system showing significant features.

The River Chelt rises close to the foot of the Cotswold escarpment east of the town of Cheltenham. It flows in a westward direction being impounded soon after its spring sources in the Dowdeswell Reservoir NGR SO 990 198. Dowdeswell reservoir is a minor source of drinking water for the Cheltenham area and is stocked as a trout fishery. Water quality at this point is good, being essentially spring water from the oolitic limestone of the Cotswold hills augmented by surface water from wooded or pasture land in the area. Nutrient levels particularly nitrogen are low (mean ammonia as N and oxidised nitrogen as N being quoted by Severn Trent Water Authority as 0.03 and 4.47 mg/l respectively). Less than 2 Km downstream, the river encounters the urban area of Cheltenham with the first sampling point at Sandford Mill Road about 2 Km further downstream at NGR SO 957 214. At this point the stream would be expected to be clean with good water quality, a diverse macroinvertebrate fauna and supporting a salmonid fishery. Data provided by the Water Authority suggest that there is an increase in mean ammonia and BOD levels over that at the

Fig 2·1 River Chelt - the system studied



Dowdeswell Reservoir but that apart from occasional high figures the quality for most of the time is good. These occasional high results are indicative of the effects of urban run off and may be incidents such as overflows from blocked foul sewers or accidental spillages.

From Sandford Mill Road the river runs through the central axis of the town of Cheltenham. In the town centre the stream runs in culvert and for half of its course in open channel is in public parks. After emerging from the urban area there are some 2 Km of open country with mostly pasture before the next sample site at Withy Bridge NGR SO 905 247 is reached. In passing through the urban area the river has its nature and background quality altered. Whilst there are no known significant regular polluting discharges to the river in this reach there are an unknown number of inputs of surface water from the town. These range from drains from single properties to surface water sewers serving large mixed catchments. There are possibilities for localised connections of foul sewage to these nominally surface drainage sytems and the strong likelihood of uncontrolled discharges of waste water from industrial premises containing oil, organic matter, detergents, cooling water or other chemicals. Any point discharges of sufficient volume or polluting potential to cause measurable deterioration in downstream river quality would most likely have been brought to the attention of the Water Authority after which action would be taken to bring such discharge under control. The other individually less significant sources of pollution would have, when taken together, either in dry or wet weather, an overall

effect of a diffuse or non point source of pollution which would show up in regular sampling. In addition the sewerage system in parts of Cheltenham is in excess of 150 years old and adds further sewage input particularly in wet weather. Hence the water quality at Withy Bridge should still be reasonably good but will reflect the urban area input. Mean figures for the period 1/1/80 to 1/2/86 are 0.58 mg/l ammonia as N; 5.26 mg/l total oxidised nitrogen as N and 4.2 mg/l BOD (Severn Trent Water Authority data).

The next sampling point at Boddington Bridge NGR SO 895 259 is less than 2 Km downstream of Withy Bridge but 750 m upstream of Boddington Bridge there is the outfall of the treated effluent from the Water Authority's Hayden water reclamation works which serves Cheltenham and some outlying districts. This consented and fully treated effluent has a dominating effect on the physical, chemical and biological character of the river for the entire 7 Km of its remaining length before its confluence with the River Severn. The main reason, for this effect is the volume of effluent being high in relation to the natural streamflow. For instance at times the effluent can be in excess of 80% of the combined flow downstream. Data on chemical quality reflect this effect and with ammonia mean 5.16 mg/l as N; total oxidised nitrogen 13.32 mg/l as N and BOD 10.6 all at least 90 samples (Severn Trent Water Authority) over the period 1/1/80 to 1/2/86, there is a marked contrast to the Withy Bridge quality. Nevertheless the combined river water and effluent has an open channel in which a natural ecosystem can develop subject to the environmental factors of temperature, channel morphology, geology and hydrology.

A further site on the River Chelt at Inchmoor Bridge NGR SO 869 250 is about 4 Km downstream of Boddington Bridge and because the channel character is maintained between the two sites, represents the changed chemical and biological status resulting from the self-purification in 4 Km of river.

For the catchment as a whole, away from the Cotswold Scarp, the surface gradients are generally slack and although the natural drainage is not good the paved areas tend to cause flashy conditions in the river with tendency to flood. The predominant soils in the area are non-calcareous clay types although there are some sandy gravel areas associated with the river terraces. At the Boddington Bridge site the catchment area is about 30 Km^2 with more than 35% urban area. Of the non urban area most is pasture land with some cereal growing. There are few intensive animal rearing units in the catchment area and this may have a bearing on the input of allochthonous organic matter and bacteria.

Major sites for sampling were the River Chelt at Withy Bridge and Boddington Bridge. Other sites chosen to cover a wider range of conditions or to complement other sites are listed below with brief details in Table 2.1 Of the total list of sites about half could be classed as dirty and the number of samples taken from dirty and clean sites was roughly equal.

Table 2.1

LIST OF SAMPLE SITES

Site	NGR	Samples	Description
R Chelt Sandford Mill	SO 957 214	12	see text
R Chelt Withy Bridge	SO 905 247	24	see text
R Chelt Boddington Bridge	SO 895 259	34	see text
R Chelt Inchmoor Bridge	SO 869 250	17	see text
Hayden WRW final effluent	SO 906 229	13	Biological filter works
settled sewage	SO 906 229	1	treating mixed domestic and industrial
filtered effluent	SO 906 229	1	sewage; effluent partly polished
R Severn Tewkesbury	SO 889 337	7	Clean lowland river potable water supply
R Leadon u/s Ledbury	SO 700 373	2	Clean, agricultural catchment
R Leadon d/s Ledbury	SO 701 368	4	Affected by Ledbury sewage effluent
Ledbury WRW final effluent	SO 701 369	4	Filter plant good quality effluent
Ledbury WRW filter effluent	SO 701 369	3	Receives Jam making effluent
R Avon Tewkesbury	SO 893 332	3	Class 2 coarse fishing lowland river
R Swilgate Tewkesbury	SO 889 322	1	Clean river receives treated sewage effluent
Tewkesbury WRW final eff	SO 883 318	1	Fully treated but only moderate quality
Ripple Brook Mythe	SO 886 342	1	Clean country stream
R Frome u/s Creamery	SO 790 053	1	High class trout fishery spring fed
R Frome d/s Creamery	SO 783 056	1	Some effect from Creamery
Creamery treated effluent	SO 797 059	1	Biological treatment but bad effluents at times
Longford WRW final effluent	SO 848 210	2	Activated sludge plant
settled sewage	SO 848 210	2	high ammonia but low
mixed liquor	SO 848 210	2	BOD. Mixed domestic and industrial sewage

2.2 ENZYME ASSAYS

In order to ensure a minimal risk of raw data being lost or confused a system of sample registration was used. Each sample was assigned a unique number and essential information on date, time, site conditions was recorded. All analysis results and details of sample registration numbers, analytical conditions, sample size or dilution and comments arising from the analysis were recorded on a work sheet one of which was designed for each assay or determination.

2.2.1 Protease Assay

2.2.1.1 Standard Assay

For the present study a method based on casein substrate as used by Batistic et al (1980) was adopted. Assays were carried out in 10 ml graduated borosilicate glass stoppered tubes. All glassware was cleaned in a low phosphate surfactant solution (Lipsol); rinsed at least 3 times each in tap water and glass distilled water.

A solution of isoelectric casein (Difco) was made up in distilled water at 2.5 g/100 ml. Problems were encountered producing this solution because of the tendency of casein to form almost insoluble lumps on contact with water. To avoid problems cold water was used with a magnetic stirrer creating a vortex when the powdered casein was slowly added to the water. A few drops of 5N sodium hydroxide was added to aid solution and this had a minimal effect on the final pH of the casein solution.

The assay buffer (TTA tris-tes-acetic acid) contained: Tris (tris [hydroxymethyl] amino methane) 3 x 10^{-2} M; Tes (N - tris [hydroxymethyl] - methyl - 2 - amino - ethane sulphonic acid) 3 x 10^{-2} M and acetic acid, 3 x 10^{-2} M. (Flint and Hopton, 1976). This buffer can be used over a very wide range typically pH 4-11 and can be sterilised by autoclaving at 121°C for 15 mins. The protease assay was routinely carried out at pH 9.0 the optimum activity pH.

As the assay is based upon the production of blue black colour on reaction of TCA (trichloroacetic acid) - soluble tyrosyl derivatives with Folins reagent, a calibration of standard tyrosine solutions was carried out. Tyrosine was made up at 0.5 g/100 ml (based on the expected release of tyrosine for complete hydrolysis of the amounts of casein used in assays) in distilled water with a few drops of 5N sodium hydroxide added to aid dissolution. 1 ml of Folins Reagent (diluted 3 fold with distilled water) was added to known concentrations of tyrosine in duplicate.

The tubes were well mixed, allowed to stand at room temperature for 15 mins to allow complete colour development. Absorbance was recorded at 750 nm using a CE272 spectrophotometer (Cecil Instruments) Fig 2.2 shows the calibration curve. Over the range of 0 to 25 µg/ml tyrosine there was a good linear relation ($r = 0.998$ P 0.001).

Fig 2·2 Calibration of Folin's method for Tyrosine

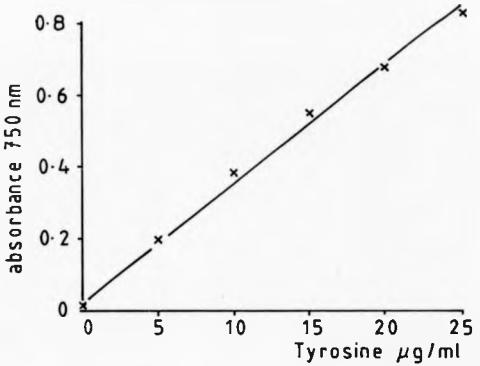
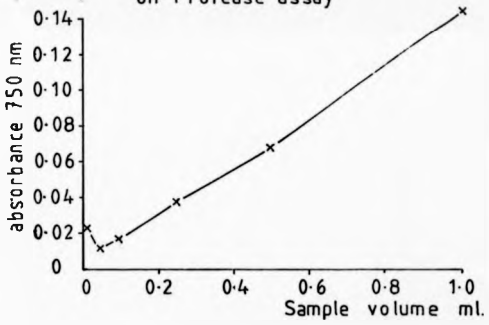


Fig 2·3 Effect of sample size on Protease assay



All buffer, substrate and tyrosine standards were stored in small volumes at $-20^{\circ}C$ until needed.

Assays were set up adding to the tubes 200 µl buffer, substrate at a concentration expected to leave unchanged casein at the end of the assay to avoid underestimation of activity; usually 200 ul and sample usually 1 ml but smaller volumes for samples expected to have high activity. Reaction mixture was made up to 2 ml with distilled water and 0.2 ml of toluene was added to prevent bacterial growth and utilisation of protein hydrolytic products at the relatively extended incubation periods employed to obtain measurable breakdown of the casein (Verstraete et al 1976).

The tubes were incubated in a water bath at $35^{\circ}C$ for periods up to 72 hours. 1 ml of 17.5% (W/V) TCA solution was added to terminate the reaction. Distilled water was added to make up the volume to 10 ml and this mixture was centrifuged at low speed, 2000g, in a M.S.E. bench centrifuge for 10 minutes. 4 ml of the clear supernatant was transferred to a clean tube. 1 ml 10% sodium hexametaphosphate (to prevent interference from calcium and other cations), 0.5 ml 5N sodium hydroxide (to neutralise TCA and raise pH to a suitable level for the addition of Folins reagent) and finally 1 ml of Folin-Ciocalteau reagent (diluted three fold with distilled water) were added in that order.

The tubes were mixed well and allowed to stand at room temperature for 15 minutes to allow complete colour development. Absorbance was recorded at 750 nm.

Four blanks were set up:

i) sample, no substrate added;

ii) substrate, no sample added;

iii) buffer no sample or substrate;

iv) distilled water.

Activity was calculated by comparing absorbance of the test (corrected for substrate, sample and buffer blanks) with the tyrosine standard (corrected for water blank) and expressed as ng tyrosine released/ml sample/hour.

An overall check on the reproducibility was carried out by setting up 20 replicates of the standard assay, measuring absorbance increase after 48 hours incubation. The coefficient of variation of the results was 7%. The exclusion of the results from 4 tubes of a different form (subsequently only round bottomed tubes were used) decreased the coefficient of variation to 5.3% which was considered an acceptable level of precision.

2.2.1.2 Effect of Sample Volume

Standard assay conditions were set up but varying sample volumes so that for the substrate concentration of 2.5 mg/ml casein as used by Batistic et al (1980) the effect of different amounts of added enzyme could be measured.

Fig 2.3 shows that there was a change in activity directly proportional to sample volume for sample volumes from 1 ml to about 0.1 ml. For sample volumes less than 0.1 ml the linear relationship did not hold but this may have been due to the inaccuracies of the techniques at low activities linked with scaling errors on dilution. Sample volumes less than 0.1 ml were only used very infrequently (usually 1 ml of a suitable serial dilution was used).

2.2.1.3 Effect of Incubation Time

The course of the reaction was followed by setting up a standard assay with replicates which were incubated for varying times up to 98.5 hours.

Fig 2.4 shows that enzyme activity increases linearly with incubation time up to 22 hours when the rate fell. For the full incubation time there remains a linear relation between absorbance increase and time.

2.2.1.4 Effect of pH on Enzyme Activity

The effect of pH of the reaction mixture was measured by setting up a series of standard assays in which the pH was varied from pH 7 to 11. Fig 2.5 shows the pH profile of activity expressed as a percentage of the maximum activity.

Fig 2·4 Time-course Saccharase & Protease assays

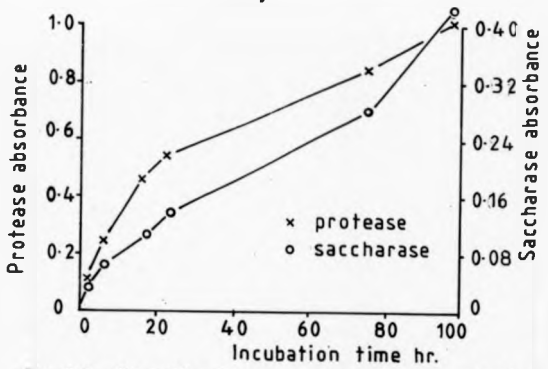
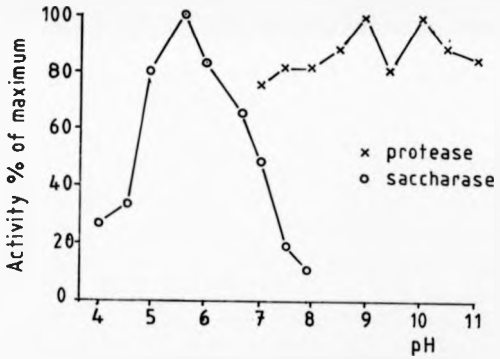


Fig 2·5 pH profile Saccharase & Protease activity.



There was little change in activity with a peak at pH 9 and a similar one at pH 10.1. A pH of 9.0 was selected for routine assay on the basis that pH 9 was nearer to ambient pH in sites sampled. The existence of 2 peaks suggested more than one enzyme was present in the sample measured.

2.2.1.5 Effect of Incubation Temperature on Enzyme Activity

The effect of temperature of incubation was measured by setting up a series of standard assays which were incubated in water baths with temperatures ranging from 20°C to 65°C. Fig 2.6 shows the temperature profile of activity expressed as percentage of the maximum activity.

Similarly to the pH profile there was little change with the temperature with maximum activity at 45°C. 35°C was chosen as the standard temperature of incubation for practical reasons and there was negligible loss of activity at the lower temperature.

2.2.2 Saccharase Assay

Saccharase action results in the hydrolytic splitting of the 1, 2 - α, β - linkage to produce one molecule each of glucose and fructose both of which are reducing sugars. Measurement of reducing sugars was the basis of the method of Veratraete et al (1976) and is used in this study. The method for measurement of reducing sugar is a copper reduction method described by Spiro (1966). Solutions of reducing sugars are heated to boiling with

Fig 2·6 Temperature profile Saccharase & Protease activity

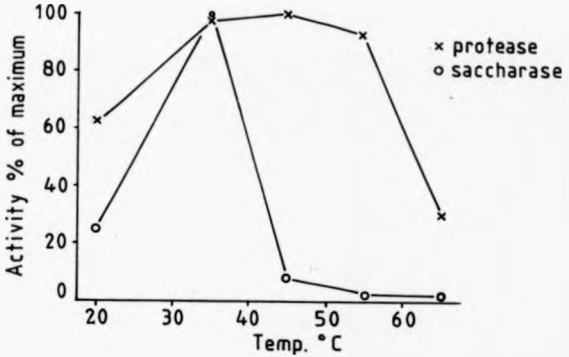
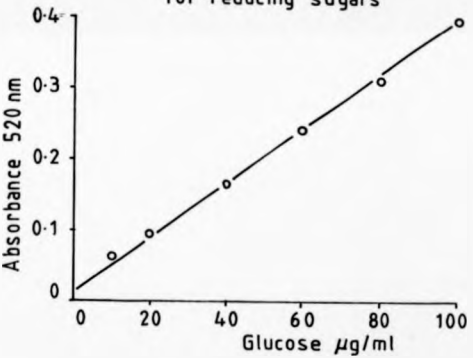


Fig 2·7 Calibration of Nelson-Somogyi method for reducing sugars



added alkaline copper reagent of the following composition:

Anhydrous disodium hydrogen phosphate 28g,

Rochelle salt (potassium sodium tartrate) 40g

dissolved in 700 ml distilled water then the following added:

100 ml N sodium hydroxide

80 ml 10% solution of copper sulphate pentahydrate; and

anhydrous sodium sulphate 180g

when all dissolved the solution was made up to 1 litre and allowed to stand for 2 days. If any sediment was visible the clear supernantant was decanted and stored at 37° in an incubator to prevent crystallisation of salts.

Solutions of sugars and copper reagent were boiled for a period of time dependent on the reducing sugars present; 10 min for glucose up to 30 min for mannose. After cooling an appropriate volume of a reagent containing ammonium molybdate and sodium arsenate made up and diluted with 1.5 N sulphuric acid, is added. The arsenomolybdate reagent was made up by dissolving 25g ammonium molybdate in 450 ml distilled water then carefully adding 21 ml concentrated sulphuric acid with stirring. Finally 3g sodium arsenate ($Na_2HAsO_4.7H_2O$) dissolved in 25 ml water was added. This mixture was incubated at 37° for 48 hours then stored at 5° in the dark. The reduced copper produces a blue colour with this reagent, which is measured after 15 min at 520 nm. The use of the alkaline copper and arsenomolybdate reagents is known as the Nelson-Somogyi method.

2.2.2.1 Standard Assay

Saccharase assays were set up in the same way as described for protease above with pH 5.5 TTA buffer.

Sucrose solution was added as substrate to give a final concentration of 1% which compares with the figure of nearly 3% used by Verstraete et al (1976) and is much higher than would be expected in any natural waters. Toluene was added as a bacteriostatic chemical.

After incubation for up to 72 hours at 35° in a water bath the reaction was stopped by the addition of 40 ul 5N NaOH to reduce acidity of mixture and 1 ml of alkaline copper reagent and immersion in a boiling water bath for 10 minutes. The glass stoppered tubes helped to prevent reoxidation of copper II oxide. After heating the tubes were cooled in cold water for 5 min and then 1 ml of arsenomolybdate reagent added. The arsenomolybdate reagent was stored in the dark at 5°. In contrast the alkaline copper reagent which has a high content of salts including sodium sulphate was stored at 37° to prevent crystallisation of the salts. Both solutions were remade if blank values started to increase.

The test solutions were made up to 10 ml with distilled water mixed, allowed to stand at room temperature for 15 min to allow full colour development and their absorbance at 520 nm read on a spectrophotometer.

An equivalent set of blanks to those used for the protease assay were set up. It was noted that the substrate blank was often significant compared with the test readings for samples with low activities.

The test absorbances were compared with standard glucose solution at 10 µg/ml. Activities were calculated from corrected absorbances and expressed as

ng reducing sugar released/ml sample/hour.

Calibration of the Nelson-Somogyi reagent for measuring glucose was carried out on replicates of a range of standard concentrations of glucose. Fig 2.7 shows a clear linear relationship between absorbance and glucose concentration up to 100 µg/ml. ($r = 0.999$ $P < 0.001$).

A test of the reproducibility of the saccharase assay was carried out by setting up 20 replicates of the standard assay using the results to calculate a coefficient of variation of 3.6% which was considered to be acceptable.

The assay was standardised in the same way as protease to check the possible effects of substrate concentration; time, pH and temperature of incubation.

2.2.2.2 Effect of Substrate Concentration

A series of standard assays were set up varying the substrate concentration between 0.01 and 1%.

Table 2.1 indicates that, at the range of sucrose substrate concentrations used, saccharase activity is not affected in a systematic way. Hence substrate concentration is not limiting under the conditions of the standard assay.

2.2.2.3 Effect of Incubation Time

The course of the reaction was followed by setting up a standard assay with replicates which were incubated for varying times up to 98.25 hours. Fig 2.4 shows a relationship between incubation time and absorbance increase (corrected for the effects of blanks).

The absorbance increase suggests a variability in activity with a slightly higher initial rate. For incubation periods in excess of 16 hours the rate is more comparable taking into account the likely variability in the test. Incubation times need to be greater than 24 hours generally to obtain measurable breakdown of the substrate.

2.2.2.4 Effect of pH on Activity

The effect of pH on activity was measured by setting up a series of standard assays with pH ranging from 4.0 to 8.0. Fig 2.5 shows the pH profile of activity expressed as a percentage of the maximum activity. Maximum activity was noted at pH 5.5 which was taken as the pH for the standard assay.

Table 2.2

EFFECT ON SACCHARASE ASSAY OF VARIATION IN SUBSTRATE CONCENTRATION

Substrate Concentration %	Corrected Absorbance Increase
1	0.067
0.5	0.071
0.25	0.025
0.1	0.040
0.05	0.034
0.01	0.022

/

what does this mean

2.2.2.5 Effect of Incubation Temperature on Enzyme Activity

The effect of temperature of incubation was measured by setting up a series of standard assays which were incubated in water baths with temperatures ranging from 20°C to 65°C. Fig 2.6 shows the temperature profile of activity expressed as a percentage of maximum activity. A sharp peak of activity was noted at 35°C which was the temperature taken for standard assays.

2.3 BACTERIAL COUNTS AND IDENTIFICATION

2.3.1 Viable Counts

2.3.1.1 Sampling

Samples for viable counting were taken in sterile Universal bottles which were held under the water surface to fill, avoiding any vegetation or debris. Processing of the samples was invariably within 2 hours and the samples were stored at 5° prior to processing.

2.3.1.2 Dilution of Samples

The samples were serially diluted in sterile Universals using a diluted phosphate buffer (potassium dihydrogen phosphate 1.36 g/l and disodium hydrogen phosphate 1.42 g/l adjusted to pH 7 autoclaved at 121°C for 15 min). Dilutions were made taking into account the degree of pollution of the sample with the object of obtaining an countable number of colonies.

Samples and dilutions were shaken vigorously by hand to distribute as far as possible the cells that might be aggregated; no attempt was made to investigate methods of improving the completeness of dispersion of aggregated bacterial flocs.

2.3.1.3 Plating Medium

0.1 ml of the dilution was pipetted onto each of 5 plates containing 20 ml of Casitone-Glycerol-Yeast Extract (CGY) Agar (Pike et al. 1972) which had been allowed to overdry by being stored at 5^{o} for up to 2 weeks. The CGY agar had the composition: Casitone (Difco), 5 g/l; Glycerol 5 g/l; Yeast Extract (Oxoid) 1 g/l and Bacto agar 15 g/l, pH adjusted to 7.2 and sterilised at $121^{o}C$ for 15 min.

The sample volume was spread over the plate with glass spreaders previously dry heat sterilised at $175^{o}C$ for 2 hours. The plates were incubated at $20^{o}C$ for 5 days when the colonies were counted. Counts were expressed as colony forming units/ml.

Reproducibility of the viable counts were checked with 15 replicate dilutions of a sample of the River Severn at Tewkesbury diluted 2×10^{2} and plated onto 5 plates. A coefficient of variation of 10% was obtained but whilst this is high the replicates showed a higher than normal number of plates with spreading colonies and combined with the large number of colonies on the plates this coefficient of variation would normally be expected to be much lower.

2.3.2 Differential Colony Counts

Natural waters tend to have bacterial flora that are heterogeneous with respect to both numbers and taxa. Attempts have been made by some workers to characterise the metabolic repertoire of isolated bacteria and to count various classes in order to relate those numbers to various limnological parameters including enzyme activity (Hankin and Sands, 1974; Nitkowski et al. 1977; Rawlings and Wood, 1978).

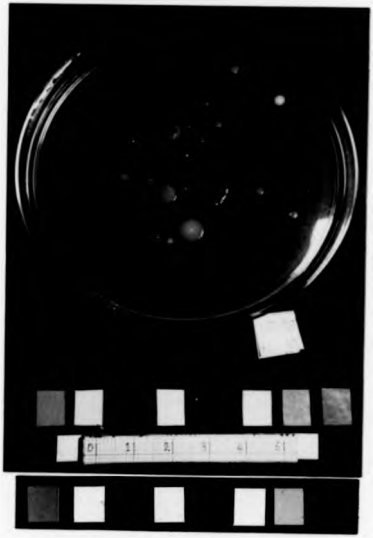
In the present study differential counts were made on the plates used for viable counting based on the physical appearance of the colonies. Colonies were categorised on a subjective basis into one of 6 groups. The groups reflected the frequency with which the various types of colony were seen on the plates. The main support for this differential count was the ease with which orange pigmented colonies could be distinguished. It would have been possible to simply count orange/yellow pigmented colonies and others but it was decided that an effort should be made to subdivide the non-pigmented colonies. It is accepted that the subdivision is subjective and that colonies may be counted in the wrong category hence masking any underlying link with enzyme activity but it was judged a worthwhile exercise if there was a chance of detecting a more specific link between a class of bacteria and enzyme activity. Such a link could easily be missed in a comparison of measured enzyme activity and gross viable count where a non producing bacteria could in an unpredictable way swamp the plate count.

In order to carry out the differential count in as consistent a manner as possible photographs were taken of the plates to be counted so that overall comparisons could be made of colony appearance and pigmentation. Unfortunately this exercise was only planned and carried out towards the end of the study so a limited number of results were obtained. To compound the variability in what was a small sample of results the samples tended to be from a range of sites encompassing clean and dirty rivers and effluents. It would have been preferable to have enough results to compare sites as has been done for other determinands but pooling of data has been necessary to have enough data upon which to carry out the range of statistical procedures.

Categories chosen based on colony appearance were, bearing in mind the effect on colony size and appearance of differences in such factors as incubation time and temperature and degree of plate overdrying, orange, large slimy, rough non circular, dense white round, translucent round and others. Plates 2.1 to 2.3 give examples of the photographs of the plates used for the counts.

Photographs were taken with an Olympus OM10 single lens reflex camera with a 50 mm standard Olympus lens to which was attached a x4 Hoya close up lens. An aperture of F8 was used at an exposure of 1/60 sec. The camera was tripod mounted above the plates to be photographed, which were placed on a matt black background. Lighting was by electronic flash attached to the camera and tilted in to give maximum illumination. A centimetre scale and colour patches are included for comparison. The film

Plate 2·1 Sample 183/85 plated on CGY agar



Key to colony type

1	Orange	counted together
2	Yellow/Pink	
3	Large slimy	
4	Rough non-circular	
5	Dense white round	
6	Translucent	

Plate 2·2 Sample 184/85 plated on CGY agar

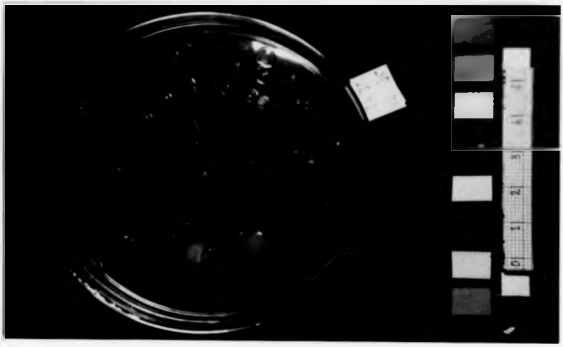
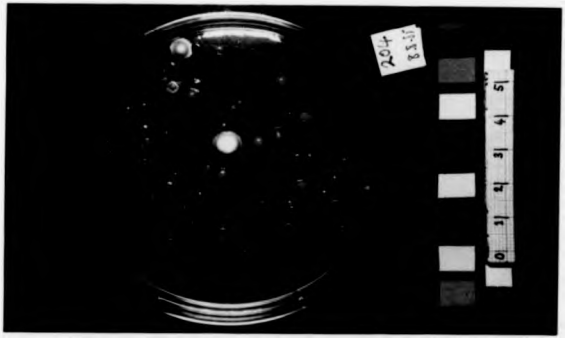


Plate 2·3 Sample 204/85 plated on CGY agar



key to colony type see plate 2·1

used was Kodak Kodacolour VR 100 processed by Supasnaps Ltd.

2.3.3 Identification Methods for Bacteria Isolated from Natural Waters

Isolates of the bacterial flora from the natural waters were identified to at least a generic level.

i) Gram staining This was carried out by the Jensen modified method (Cruickshank et al 1968) on a heat fixed smear of a 48 hour CGY agar culture emulsified in sterile distilled water. The smear was flooded with crystal violet for 1 min fixed with Lugol's iodine for 30 sec and decolourised with acetone until no more blue colour was removed. After rinsing with distilled water the smear was counterstained with the basic red dye Saffranin for 1 min, dried and examined under oil immersion.

ii) Motility This was tested on a loopful of 48 hour culture grown in CGY broth (the CGY medium without added agar). The drop of liquid was placed on a cover slip and inverted over a cavity slide and examined under a phase contrast microscope (Wild Instruments). Controls were set up using Bacillus (non motile) and Pseudomonas (motile) cultures.

iii) Kovacs' Oxidase The possession by an organism of the cytochrome oxidase enzyme system was checked using the redox dye tetramethyl-p-phenylenediamine dihydrochloride known as Kovac's reagent (Kovacs, 1956).

The reagent was made up freshly at 0.1 g/10 ml in distilled water. Strips of GFC filter papers were soaked in the solution and small amounts of 48 hour CGY agar culture were spread on the filters using a platinum loop.

The appearance of a blue-purple colour within 10 sec was marked as a positive result. Pseudomonas fluorescens was used as a control.

iv) Oxidative and Fermentative Breakdown of Glucose The oxidative or fermentative breakdown of glucose in a soft agar medium was tested by the method of Hugh and Leifson (1953). This test is essentially for gram-negative organisms and a modification for gram positive organisms was not considered necessary in this study.

Oxidation-Fermentation agar medium (Difco) was made up at 9.4 g/l in distilled water, sterilised at 121° for 15 min. A separately sterilised (autoclaving 121° for 15 min) glucose solution was added aseptically to give a final concentration of glucose of 0.5% (W/V). Medium was dispensed into 10 cm x 1 cm tubes to a depth of 6 cm. Duplicate tubes were inoculated by stabbing with a straight wire from CGY broth subcultures of the unknown organisms. One of the tubes was sealed with a layer of sterile melted petroleum jelly to a depth of 5-10 mm. Tubes were incubated at 20°C for up to a week checking growth at intervals. Controls were set up using Pseudomonas fluorescens (oxidative), E Coli (fermentative) and Bacillus cereus var mycoides (gram positive control).

After incubation a fermentative action is indicated by a yellow colouration throughout both tubes; oxidative action is shown by an acid reaction at the surface of the open tube only. Any blue colour at the surface of the tube denotes an alkaline reaction probably caused by deamination of amino acids in the medium.

Gas production would be indicated by bubbles in the medium.

v) Carbohydrate Utilisation This was investigated by inoculating tubes of peptone broth (peptone (Oxoid) 10 g/l, sodium chloride 5 g/l, phenol red indicator 0.4% W/V aqueous solution, adjusted to pH 7.5 autoclaved 121° for 15 min) supplemented with additions of sterile carbohydrates to give final concentrations of 1% w/v. The carbohydrates used were: lactose, maltose, sucrose, glucose and mannitol. Positive results were shown by a yellow colouration (denoting acid production) and growth. No controls were set up as this test was to record the extent of carbohydrate utilisation.

vi) Citrate Utilisation This was included as a test for possible coliforms. Simmons citrate agar medium (Simmons, 1926) containing NaCl 5 g/l, $MgSO_4.7H_2O$ 0.2 g/l, ammonium dihydrogen phosphate 1 g/l, dipotassium hydrogen phosphate 1 g/l, citric acid 2 g/l, agar 1.2% and bromothymol blue indicator; autoclaved 115°C for 10 min, was poured as slopes in Universal bottles. Inoculation was from 48 hour CGY broth cultures and after incubation

at $20^{o}C$ for up to a week positive utilisation was denoted by a colour change from pale green to blue.

vii) Catalase Activity This was checked by placing a drop of 100 vols hydrogen peroxide on a glass slide adding a speck of 48 hour CGY agar culture. A positive result was indicated by the generation of bubbles of oxygen. Pseudomonas fluorescens was used as a positive control.

viii) Kanamycin Resistance Resistance to the antibiolic Kanamycin is shown by certain bacteria including the Flavobacterium group which are commonly found in natural waters. The unknown organisms were streaked onto nutrient agar plates containing 50 ug/ml of Kanamycin. Plates were incubated at $20^{o}C$ for 5 days when the extent of growth was noted. A Flavobacterium species was streaked as a control.

ix) Kings Medium This was used to selectively test for certain Pseudomonad species by enhancing the production of fluorescent pigments by those species.

Kings medium A (King, et al 1954) comprised Peptone 20 g/l, glycerol 10 ml/l anhydrous potassium sulphate 1.4 g/l and Bacto agar 15 g/l.

Kings medium B comprised Proteose-peptone 20 g/l; glycerol 10 ml/l; dipotassium hydrogen orthophosphate 1.5 g/l; magnesium sulphate ($Mg\ SO_4\ 7H_2O$). 1.5 g/l and Bacto agar 15 g/l.

Medium were autoclaved for 15 min at 121°C then poured into plastic petri dishes.

Overdried plates were streaked with unknown cultures and Pseudomonas aeruginosa and Pseudomonas fluorescens were used as controls being producers of the pigments pyocyanine and fluorescein respectively.

2.4 CHEMICAL METHODS AND FLOW MEASUREMENT

To complement and set a context for enzyme activity measurements, conventional chemical quality determinands were also measured on water samples. These were: dissolved oxygen, temperature and biochemical oxygen demand (BOD), suspended solids and ashed suspended solids, and a range of determinands grouped together comprising ammonia, total oxidised nitrogen, pH, chloride and electrical conductivity, which were measured by an auto-analyser at the Malvern Laboratory of the Severn-Trent Water Authority. These methods are discussed below.

2.4.1 Dissolved Oxygen and Temperature

Dissolved oxygen and temperature were measured on site using a portable dissolved oxygen meter (Model 57 Yellow Springs Instruments).

2.4.2 BOD

BOD was measured by the Dilution Metod described in the Department of the Environment approved methods (Anon, 1972)

250 ml narrow necked glass bottles were used. Samples were measured undiluted for clean rivers (where BOD was expected to be 5 mg/l or less) or diluted with synthetic dilution water for dirty river or effluents. Dilution water is formulated to duplicate the qualities of a very clean natural water and is made up with distilled water with additions of trace elements: 1 ml each of reagents ferric chloride (0.125 g Fe Cl_3/l $6H_2O$); calcium chloride (27.5 g $CaCl_2$/l); magnesium sulphate (25 g/Mg $SO_4.7H_2O$/l) and phosphate buffer (42.5 g KH_2/l, 8.8 g Na OH and 2 g $(NH_4)_2$ SO_4). Dilution water was aerated with compressed air for 30 min before us.

The 250 ml bottles were carefully filled with the sample any bubbles being allowed to escape. To suppress nitrification in the bottles 0.25 ml of a solution of 0.5 g/l allylthiourea was added to each bottle before stoppering.

One of the bottles had its dissolved oxygen content measured immediately using the Winkler Method. 1 ml manganous sulphate (500 g Mn $SO_4.5H_2O$/l) was added then 1 ml alkaline iodide-azide solution (40 g NaOH; 900 g NaI; 19 g NaN_3 per litre). Bottles were carefully stoppered to avoid trapping bubbles mixed by inversion and the brown precipitate of hydrated manganese oxide allowed to settle. Iodine was released from the sodium iodide by acidifying with 2 ml syrupy phosphoric acid. After mixing and allowing the iodine solution to develop the iodine was titrated with standard N/40 sodium thiosulphate solution, using soluble starch solution (5 g/l) as an indicator.

The second bottle was incubated in the dark for 5 days at 20°C. Dissolved oxygen measurements were then carried out and the difference in initial and final readings taking account of blank values of dilution water and the actual dilution if any of the sample gives the BOD in mg/l.

Reproducibility of this procedure was checked with 20 replicate determinations giving a coefficient of variation of 12.7% for total ATU BOD which is high but acceptable considering the known varibility inherent in this test.

2.4.3 Suspended Solids

Portions of sample usually 500 ml for clean samples and less for dirty rivers and affluents or where colloidal solids were evident were filtered through preweighed washed and ashed 70 mm dia GF/C filter papers (Whatman) using a 3 component Hartley funnel under suction from vacuum pumps driven by mains water pressure.

Filters were dried at 105°C for 1 hour then allowed to cool in a dessicator after which they were reweighed. Dried and weighed filters were ignited in a muffle furnace at 500°C for 1 hour then cooled again in a dessicator and reweighed.

Weight differences and allowance for sample size gave concentration of total suspended solids and non volatile suspended solids on ignition.

Reproducibility checks on 20 replicate determinations of 500 mls of sample gave an acceptable coefficient of variation of 3.4% for total suspended solids and 3.2% for ashed solids.

2.4.4 Autoanalysis Determinands

Autoanalysis equipment (Technicon instruments) was set up at the Malvern Laboratory of the Severn-Trent Water Authority to carry out rapid large sample number analysis of determinands such as ammonia, oxidised nitrogen, pH, chloride and conductivity which are part of routine "sanitary analysis". Samples from this study were prepared and added to the sample handling equipment of the machine. Output from the machine was a chart with different colour traces for the different determinands at the different analysis ranges used.

Reagents and standards were made up in bulk by Severn Trent staff who operated and serviced the equipment. All charts and results were processed separately from the Malvern Laboratory's routine work.

A range of standards were analysed each time for the sample runs and these were used to compare peak heights to work out concentrations in the samples.

The calibration of standards was consistent and with high correlation of concentration and peak height r typically better than 0.999 and coefficient of variation on replicates of a single sample or standard solution were very low typically 1%.

Colorimetric methods are employed. Ammonia in the sample reacts with alkaline sodium phenate in the presence of sodium nitroprusside as catalyst with sodium hypochlorite donating chlorine to form a blue coloured indophenol dye which is measured at 650 nm.

Oxidised nitrogen which would be present in a sample as nitrite or nitrate is initially all converted to nitrite using a copper-hydrazine solution. This nitrite is mixed with a solution of sulphanilic acid and N-1-naphthylethylene-diamine dihydrochloride in acid conditions. The resulting pink coloured diazo dye is measured at 520 nm.

Chloride ions in the sample liberate thiocyanate ions from a solution of mercuric thiocyanate. The liberated thiocyanate ions react with ferric ions (Iron III) added in the form of ferric nitrate to form a highly coloured red iron III thiocyanate compound which is measured at 480 nm.

pH is measured by a conventional combined glass electrode compensated for temperature and electrical conductivity by a conventional 1 cm cell.

The chart recorder output from the autoanalyser was used to calculate concentrations of the determinands described above by measuring peak heights of the unknowns and comparing with peak heights of added standards.

2.5 FLOW MEASUREMENT

Flow information at sites on the River Chelt was initially obtained by field gaugings. A stretch of river was sought with consistent cross section area, depth and an absence of interfering factors such as differences in substratum, for example mud and stone, excessive vegetation or presence of rubbish. The River Chelt is a fast flowing stream with a riffle type bed and occasional pools. It is fairly sinuous so these criteria were not easy to fulfil.

Having selected a gauging point current measurements were taken using an OTT current meter. A transect of the stream was made measuring current speed a minimum of 3 times at 2/3 of the depth of the flow avoiding quiescent areas. Channel depth and width measurements were taken to calculate cross-sectional area in metre^2.

Ott current meters work by counting the number of revolutions of an impellor held into the flow for a measured interval, in this case 100 seconds. Two different impellors were used to cater for a wide range of current speeds. Tables of calibration were used to work out current speed.

Flow figures were calculated by multiplying cross-section by current speed and expressed as megalitres/day (Ml/d or 10^6 l/d).

These gaugings were time consuming, not considered very accurate and having an element of hazard at high flow rates in storm conditions particularly downstream of the discharge from the Hayden water reclamation works. It was therefore decided to use flow records produced from two devices maintained by the Severn-Trent Water Authority.

Sewage flows at the Hayden works are continuously measured by a flume at the inlet to primary settlement. The flume has a float and recorder for levels from which flow is calculated by standard equations relating water level differences above and below the flume to flows. An integrator on the flow recorder provides information on total flows over a chosen period. Daily flows from this works were worked out from these integrator readings.

Downstream of the discharge from this sewage works to the River Chelt there is a river gauging station. This installation comprises a broad crested weir built into the river channel constructed in concrete. Level measurements are recorded by means of a float system and stored in a data logging system. This stage level information is retrieved periodically by employees of the Water Authority and processed by computer which uses formulae to relate stage to discharge. Flow data are archived and retrieved as needed. A chart record of stage readings is kept. Fig 2.8 is a photocopy of a typical chart record at this site and illustrates the pronounced diurnal flow pattern which reflects the discharge of sewage effluent from Hayden reclamation works 1500 metres upstream. It can be seen that instantaneous flows vary widely and would be difficult

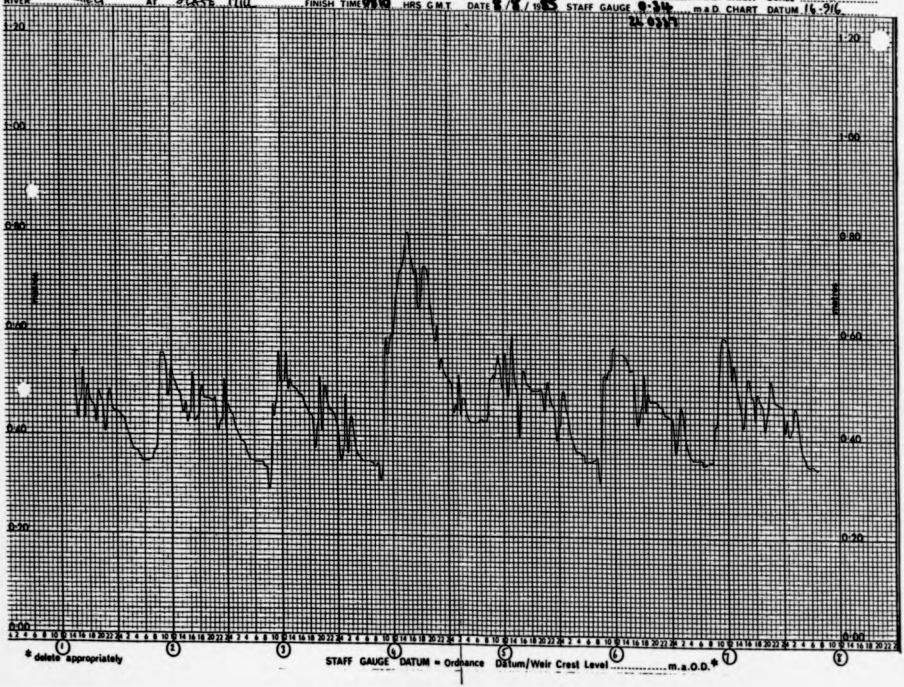


Figure 2.8 Flow record for River Chelt at Slate Mill

data to relate to the limited spot samples taken for enzyme activity, bacterial counts and other chemical measurements. Accordingly mean daily flow information was retrieved from the achived data to be used for this study.

The data on daily flows at Hayden works and the River Chelt downstream were used to calculate the daily flows at the Withy Bridge site upstream of the effluent discharge.

No attempt was made to incorporate rainfall data into this study because of the complexities of the hydrological modelling of catchments.

2.6 ADDITIONAL EXPERIMENTS ON ENZYME ACTIVITY IN ISOLATED BACTERIA

Methods detailed in the preceeding sections (2.2 and 2.3) were used for the survey of enzyme activity in natural waters. Chapter 4 describes experiments on cultures of organisms isolated from the rivers.

Standard enzyme assay conditions were used in these experiments with sample sizes dependent on the nature of the samples being assayed. Samples ranged from river water amended with the addition of sucrose or casein to broth or minimal medium cultures to crude extracts of cells grown in liquid medium.

The description of procedures for each of these experiments is presented in each of the appropriate subsections of Chapter 4. Materials and Methods that are common to all experiments in Chapter 4 are listed below.

i) Minimal Medium This medium was taken from Flint and Hopton (1976) and had a composition at working strength as follows:

Ammonium sulphate	1 g/l
Magnesium sulphate ($MgSO_4$ $7H_2O$)	10 g/l
Ferric sulphate ($FeSO_4$).$7H_2O$ with hydrochloric acid added to stock reagent to keep pH at 3)	0.0005 g/l
Calcium chloride ($CaCl_2$ fused granules)	0.1 g/l
Tris buffer adjusted to pH 7.2	0.05M
Trace element (composition listed below)	2.5 ml/l

These components of the medium are mixed and sterilised at 121°C for 15 mins.

Stock trace element (Kelly & Clarke, 1962)

$Fe\ SO_4.(NH_4)_2SO_4.6H_2O$	232 mg/l
HBO_3	464 mg/l
$CO_2SO_4.7H_2O$	191.2 mg/l
$CuSO_4.5H_2O$	16 mg/l
$MnSO_4.H_2O$	16 mg/l
$(NH_4)_2\ Mo_7\ O_{24}.4H_2O$	44 mg/l
$Zn\ SO_4.7H_2O$	348 mg/l

Hydrochloric acid is added dropwise to the trace element solution to prevent precipitate formation.

After sterilisation a separately autoclaved (121°C for 15 min) solution of potassium dihydrogen orthophosphate was added at the rate of 100 ml of 1.432 g/l KH_2PO_4 solution per litre of medium to produce a final concentration of 0.1 g PO_4/l.

Carbon sources were added aseptically before inoculation. The carbon concentration of the medium was 0.5 g C/l for glucose. This was equivalent to a concentration in the medium of 1.25 g/l glucose. Glucose solutions were sterilised separately at 121° for 15 min.

Generally the minimal medium was prepared from concentrated constituents and sterilised in volumes that on adding sterile phosphate and carbon source would make up 100 ml medium.

Additional carbon and nitrogen sources used were potassium nitrate at 1.51 g/l final concentration as a substitute for ammonium sulphate and sucrose at a final concentration of 2.81 g/l.

Particular problems were encountered in producing sterile casein solutions. As has already been indicated the preparation of aqueous solutions of isoelectric casein poses problems of failure to wet powdered casein on mixing with water and the tendency to form lumps which then take very long periods of agitation to disperse. A maximum concentration of 2% W/V was settled on to prevent problems of excessive viscosity and

foaming whilst mixing. Heat sterilisation of casein is problematic because of the risk of heat denaturation. Repeated attempts at filter sterilisation failed to reliably produce sterile solutions and finally the process of tyndallisation was adopted. Casein solution at 2% W/V concentration was dispensed in 20 ml volumes into Universal bottles which were heated to 80°C on each of 4 successive days. Failure to go turbid indicated sterilisation of the casein and also avoidance of denaturation.

Complex growth medium was Casitone-Glycerol-Yeast extract broth containing Casitone (Difco) 5 g/l; Glycerol 5 g/l Yeast Extract (Oxoid) 1 g/l made up in distilled water adjusted to pH 7.2 and sterilised at 121°C for 15 min.

Yeast extract was added to some cultures in minimal medium as a 0.1% (W/V) solution in distilled water sterilised at 121°C for 15 min. Final concentrations of yeast extract in these supplemented cultures was 0.01%.

Organisms isolated from rivers were stored at 4°C on CGY agar slopes until required. Subcultures were produced on the same medium every 4 to 6 weeks.

3 SURVEY OF ENZYME ACTIVITY IN RIVERS AND OTHER WATERS

3.1 Preliminary Examination of Data

3.1.1 Discussion of Data Set

A prime objective of this study was to measure the activity of two enzymes in aquatic environments in order to obtain information on natural levels and associated variabilities. This information could be used to compare with equivalent data on the same enzymes in other widely separated biotopes (Verstraete et al., 1976). Measurement of other properties of the waters such as biochemical oxygen demand (BOD), bacterial counts and flow would allow the investigation of relationships between enzyme activity and some of the other limnological characteristics.

Initial considerations in choosing sampling sites were based on accessibility, the ability to obtain reliable flow information and the existence of historical data held by the Severn-Trent Water Authority on determinands such as BOD and suspended solids. These data might prove a useful check on analytical techniques used for this study.

At the outset it was felt that sampling should not be restricted to a single site in order that as wide a picture as possible be obtained of in-situ enzyme activity. Accordingly two main sites were chosen on the River Chelt to provide data for the initial survey. A wider range of sites on the River Chelt and other watercourses with the addition of effluents to watercourses was chosen later and provided a data set with which to check the validity of conclusions drawn from the main sites. Early examination of the data however revealed high variability in

several determinands particularly enzyme activity and bacterial counts. This probably arose from sampling at times of environmental extremes such as very low temperatures or flood flows when the dynamic character of rivers were very evident.

It has proved to be impractical to return to the field to repeat a sampling survey under the tightly controlled environmental conditions needed to reduce natural variability. Consequently an attempt has been made to select subsets of the pooled data for the two main sites on the River Chelt at Withy Bridge and at Boddington Bridge in order to simulate sampling under conditions of greater environmental homogeneity when variability would hopefully be significantly reduced. By deciding to pool data from two sites a criticism might be levelled that it was unacceptable to treat as one population what were obviously two separate systems. The River Chelt at Withy Bridge has been described already as essentially a clean upland stream receiving occasional organic input from the Cheltenham urban area whereas the stream at Boddington Bridge which is only 3.5 km downstream of Withy Bridge is dominated by the discharge of treated sewage effluent from the Severn-Trent Water Authority's Hayden water reclamation works. Such a criticism would be warranted were any model of enzyme activity in natural waters produced from this data set, to attempt to be too generalised or to fail to take account of the limitations of the raw data. In addition it is plausible that the biological consequences of temperature variation would be comparable in a watercourse and the flow through system of a sewage works. A similar argument applies for level of organic pollution and perhaps more debatably for flow variation where considerations of transport of particles of different density and

Table 3.1

Subsets of Data Used in Preliminary Survey of Enzyme Activity

Subset Description	Number of Samples	Criteria for Subsetting	Comment
MIXED	58	Mixed all samples R Cheit at Withy Bridge & Boddington Bridge	34 samples Boddington 24 samples Withy
CLEAN	24	BOD concentration 5 mg/l was initial criterion but only two samples were greater than 5 so all samples included to maintain integrity of site.	
DIRTY	34	As for CLEAN but samples with BOD 5 mg/l chosen intially. Only 4 samples were outside this standard so all samples in fact included.	
LOW TEMP	30	Samples when temperature was equal to or less than 12° which is the median temperature for MIXED	16 samples Boddington 14 samples Withy
HIGH TEMP	28	Samples when temperature was equal to or 12.1°	18 samples Boddington 10 samples Withy
LOW FLOW	29	Samples when standardised flow was equal to or less than 172.5% which is the median flow for MIXED	25 samples Boddington 4 samples Withy
HIGH FLOW	29	Samples when standardised flow was equal to or greater than 172.6%.	4 samples Boddington 25 samples Withy

nature, apply. Initial subsetting of data is presented in table 3.1. Table 3.1 shows that using the BOD(ATU) criterion of 5 mg/l virtually all of the Withy Bridge samples would be "clean" and conversely virtually all the Boddington Bridge samples would be "dirty". A BOD(ATU) of 5 mg/l if taken as a 95th percentile is coincidentally the criterion defining the National Water Council's river Class 1B which is unpolluted or recovered from pollution. Subsetting the data based on flow produces a LOW FLOW set dominated by Boddington Bridge data. This is probably the consequence of the lower variability of flow of the effluent dominated river at Boddington Bridge resulting in more flow values being less than the median for the combined sites.

3.1.2 Frequency Distribution of Data

In carrying out a preliminary analysis of raw data, an essential first stage is to characterise, as far as possible, the underlying distribution of the property being measured. Any analysis should be linked to a continuing biological interpretation of the data. It is suggested that it is preferable to have a less sophisticated analysis that is amenable to a sensible biological interpretation as opposed to a mathematically involved model that is difficult to explain in terms of the systems and attributes being examined. Analysis usually takes the form of fitting a theoretical distribution to the empirical distribution. Subsequently the properties of the theoretical distribution are used to model the attribute under investigation. Empirical distributional patterns do not usually produce test statistics, but can give some diagnostic information about how and where data deviate from

theoretical distributions. Outlying observations are shown up in distributional plots and may be very important practically. An example would be the toxic effects to biota in rivers from episodic pollutions such as storm overflows from sewers conveying industrial sewages.

The MINITAB (Minitab Inc, Pennsylvania State University release 5.1.3 1985) statistics package used in this study produces simple histograms of data and probability plots based on a plot of variable values against their normal scores calculated by a method analogous to the calculation of standardised variables but incorporating an approximation of the distribution function of the standard normal.

The straightness of the line is taken as a measure of fit to a normal distribution. A test statistic of a product moment correlation coefficient can be used to test the hypothesis of normality. These two plots are the basis of the consideration of the distribution of data from the preliminary survey and the figures 3.1 to 3.9 illustrate the features.

Figure 3.1 shows a combination of histogram and probability plot for enzyme activity, in this case protease at Boddington Bridge. Data are not distributed smoothly but skewed and in this case there are a smaller number of higher activities detached from the main body of data. A normal probability plot is not a straight line but the correlation of activity against its N-Score (Normal Score) suggests that a null hypothesis of normal distribution of protease values at Boddington Bridge cannot be rejected at P 0.05. Log probability plotting of the same data gives a more nearly

Fig. 3·1 Histogram & Normal-Probability Plot
Protease Boddington Bridge

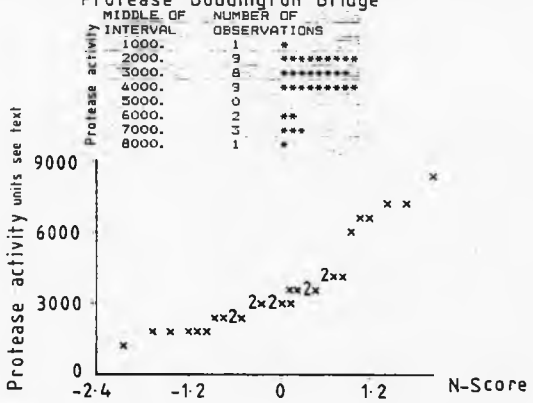
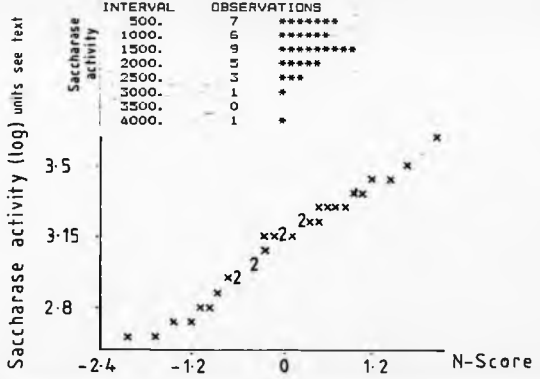


Fig. 3·2 Histogram & Log-Probability Plot
Saccharase Boddington Bridge



straight line and supports the empirical improvement achieved by log transforming these type of data. Saccharase activity at the same site illustrated in Fig. 3.2 further reinforces that view. Additionally for this activity there appears to be a threshold value for saccharase; minimum values for saccharase are not zero but a small postive value. This may reflect a lack of sensitivity in analytical methods or an apparent blank value associated with the background level in a natural water.

BOD and viable or plate count at 20° are shown in Fig 3.3 and 3.4. Both are highly skewed at the Withy Bridge site presented in these figures. Viable count in particular is far from a normal distribution. Log transformation of these two data sets straightens the probability plot sufficiently to make a log normal distribution a suitable working model.

Flow data at Boddington Bridge are plotted in Fig 3.5. This distribution is more comparable to accepted non normal patterns such as log normal or Pearson III, which involves a threshold value or a third parameter (Viessman et al. 1977). Log transformation produces a good straight line plot.

A histogram of temperature data for the "Mixed" data set in Fig 3.6 shows a much more symmetrical spread typical of temperature data.

An example of the effect of extreme values or outliers is shown in the suspended solids data at Withy Bridge presented in Fig 3.7. One value of more than 200 mg/l affects the whole probability plot. Log transformation of these data suppresses the effect of

Fig. 3·3 Histogram & Normal-Probability Plot
BOD Withy Bridge

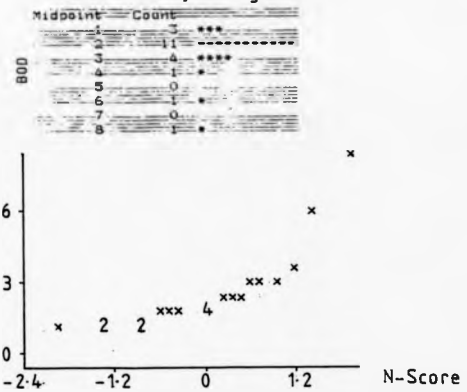


Fig. 3·4 Histogram & Normal-Probability Plot
Viable Count Withy Bridge

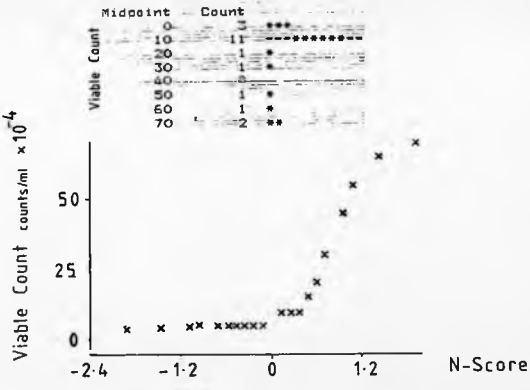


Fig.3·5 Histogram & Normal-Probability Plot
Flow Boddington Bridge

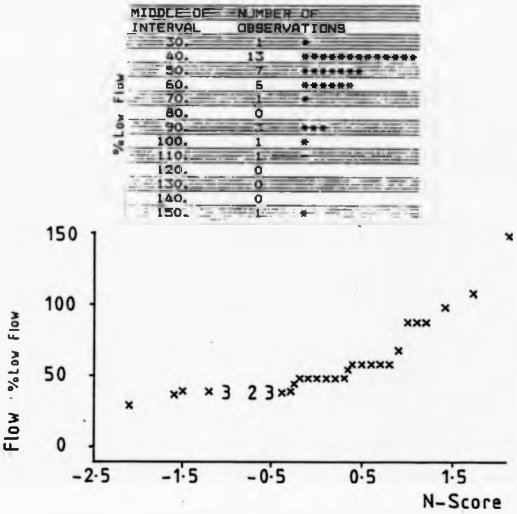
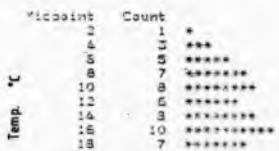


Fig.3·6 Histogram
Temp. 'Mixed'



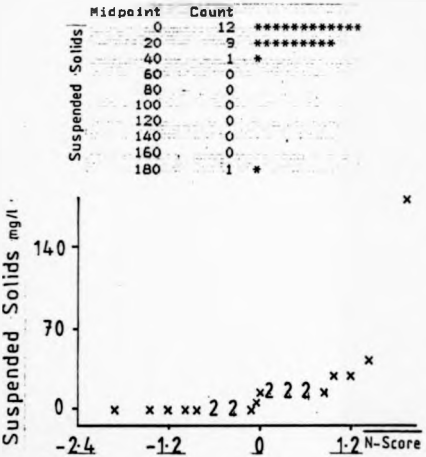


Fig. 3·7

Histogram & Normal-Probability Plot
Suspended Solids Withy Bridge

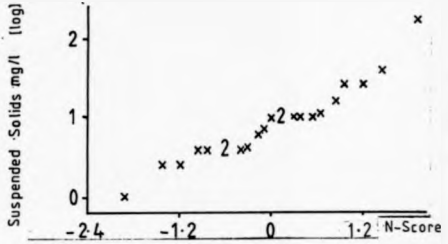


Fig. 3·8 log-Probability Plot Suspended Solids
Withy Bridge

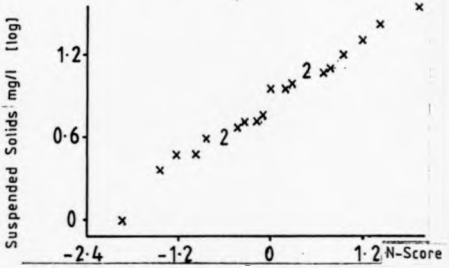


Fig. 3·9 log-Probability Plot Suspended Solids
Withy Bridge see text

this extreme result, which was a genuine result of a sample taken of the river in conditions of flood flow, as can be seen in Fig 3.8. Removal of this sample produces an even smoother fit to a log normal distribution Fig 3.9.

An overall pattern of non symmetrical distributions is noted with some histograms such as bacterial counts being either highly skewed, multimodal or even both. This complex picture has been noted by other workers (Hagenal, 1955; Montgomery and Hart, 1974; Jones, 1979 and Warn and Brew, 1980) and all point out that the use of parametric statistical methods usually requires the data to be normally distributed. A common transformation function used to achieve normality in the data is to use the logarithm of data points. The above authors suggest that empirical Normal, Log Normal or as suggested by Warn and Brew (1980), a three parameter Log Normal to take account of low but non zero values, are not necessarily theoretical distributions but they are useful working models in identifying gross effects in the aquatic environment.

Intuitively it is reasonable to suggest that limnological characters such as BOD, particulate matter and enzyme activity would be affected by a large number of separate factors. Each of these factors will have its own distribution and as suggested by Cooper and Weekes (1983) the Central Limit Theorem predicts that the combined effect on the characteristic in question would tend to cause it to be normally distributed. For conservative attributes like chloride the separate and random inputs of chloride or diluting effects of clean inputs often results in a Normal pattern. A further mechanism plays a part in such properties as BOD, bacterial count and flow; this being the non

conservative nature of such properties with their tendancy to decay. This decay function would tend to give the temporal sequence of proportionate effects which is characteristic of a Log Normal distribution. Log Normal processes are difficult to explain in physical terms and the case often cited (Ehrenberg, 1982) is of a growth function giving numbers of individuals for example that vary in a logarithmic fashion.

Water quality situations also tend to throw up occasional high values which skew the distribution; such events as storms can give exceptionally high suspended solids. These high values will have their effect on the populations reduced if a log transformation is carried out. Thus a logarithmic transformation is used for all determinands. Even where a Normal distribution seems to fit the raw data a logarithmic transformation tends to produce a better fit.

3.1.3 Summary Statistics of Data

Tables 3.2 to 3.7 present some summary statistics for the preliminary data. In almost all cases the positive skewness of the raw data is shown by the difference between the mean and the median. Generally the median is less than the mean. For BOD or Protease which is less markedly skewed the median is in excess of 70% of the mean. In contrast for Viable count a typical figure is 40% which points up the occurrence of small numbers of extreme values. Similarly flow is subject to wide fluctuation, especially in a river such as the Chelt which has an urban area as a significant part of its catchment and where rainfall tends to concentrate quickly via the sewerage system running off to give a

Table 3.2

Summary Statistics of Standardised Flow for Various Data Sets

Site	Sample No	Arithmetic Mean	Median	STD Dev.	CV STD Dev Mean x 100
BODDINGTON BR	34	163	137	74	45%
WITHY BR	24	535	337	538	101%
'LOW FLOW'	29	125	126	26.5	21%
'HIGH FLOW'	29	509	325	488	96%
'LOW TEMP'	30	446	247	503	113%
'HIGH TEMP'	28	180	128	126	70%
MIXED	58	317	173	396	125%

Units:

Ratio of mean daily flow to low 90 percentile flow expressed as percent ie

$$\frac{\text{Mean daily Flow Ml/d}}{\text{Low 90 percentile flow Ml/d}} \times 100$$

Table 3.3

Summary Statistics of Total Saccharase Activity for Various Data Sets

Site	Sample No	Arithmetic Mean	Median	STD Dev.	CV STD Dev / Mean x 100
BODDINGTON BR	32	1496	1454	828	55%
WITHY BR	22	1202	126	263	130%
'LOW FLOW'	26	1374	1454	764	56%
'HIGH FLOW'	28	592	198	870	147%
'LOW TEMP'	30	907	639	950	105%
'HIGH TEMP'	24	1046	877	848	81%
MIXED	54	969	789	909	94%

Units:

ng reducing sugar released/ml sample/hr

Table 3.4

Summary Statistics of Total Protease Activity for Various Data Sets

Site	Sample No	Arithmetic Mean	Median	STD Dev.	CV STD Dev Mean x 100
BODDINGTON BR	33	3613	3106	1809	50%
WITHY BR	21	770	732	412	54%
'LOW FLOW'	28	3345	2997	1978	59%
'HIGH FLOW'	26	1606	1085	1540	96%
'LOW TEMP'	27	2405	2000	2096	87%
'HIGH TEMP'	27	2610	2287	1854	71%
MIXED	54	2508	2094	1981	79%

Units:

ng tyrosine released/ml sample/hr

Table 3.5

Summary Statistics of BOD for Various Data Sets

Site	Sample No	Arithmetic Mean	Median	STD Dev.	CV STD Dev Mean x 100
BODDINGTON BR	27	8.5	8.0	4.1	48%
WITHY BR	21	2.5	2.0	1.6	64%
'LOW FLOW'	23	8.1	6.0	4.7	58%
'HIGH FLOW'	25	3.8	2.5	2.8	74%
'LOW TEMP'	23	4.4	3.2	2.7	61%
'HIGH TEMP'	25	7.2	5.5	5.1	71%
MIXED	48	5.9	4.9	5.5	93%

Units:

mg/l

Table 3.6

Summary Statistics of Suspended Solids for Various Data Sets

Site	Sample No	Arithmetic Mean	Median	STD Dev.	CV STD Dev Mean x 100
BODDINGTON BR	29	23.2	16.8	26.3	113%
WITHY BR	23	17.2	8.0	35.7	208%
'LOW FLOW'	24	16.1	15.0	9.6	60%
'HIGH FLOW'	28	24.4	11.0	40.5	166%
'LOW TEMP'	27	26.7	14.0	40.4	151%
'HIGH TEMP'	25	13.9	10.8	10.5	76%
MIXED	52	20.6	12.0	30.7	149%

Units:

mg/l

Table 3.7

Summary Statistics of Viable Count for Various Data Sets

Site	Sample No	Arithmetic Mean	Median	STD Dev.	CV STD Dev Mean x 100
BODDINGTON BR	27	$1.7x10^6$	$7.4x10^5$	$2.2x10^6$	129%
WITHY BR	20	$1.89x10^5$	$7.4x10^4$	$2.1x10^5$	111%
'LOW FLOW'	23	$1.87x10^6$	$6.6x10^5$	$2.34x10^6$	120%
'HIGH FLOW'	24	$2.78x10^5$	$9.6x10^4$	$2.85x10^5$	103%
'LOW TEMP'	26	$3.05x10^5$	$2.8x10^5$	$2.44x10^5$	80%
'HIGH TEMP'	21	$1.99x10^6$	$9.0x10^5$	$2.42x10^6$	122%
MIXED	47	$1.06x10^6$	$4.5x10^5$	$1.83x10^6$	173%

Units:

counts per ml

"flashy" flow pattern. Under low flow conditions the distribution of flow is approaching normal as opposed to high flow where it is highly skewed.

As to enzyme activity; there is a ten fold difference in the mean levels at the Withy Bridge as compared to the Boddington Bridge sites which are the "clean" and "dirty" sites respectively. Protease shows a similar but less marked reduction in activity going from polluted to less polluted. Subsetted data shows a proportionate effect depending on the numbers of the respective site data present.

BOD being an established indicator of organic pollution is higher at the Boddington Bridge site with a mean of 8.5 mg/l than at the cleaner site Withy Bridge; having a mean of 2.5 mg/l. A second commonly quoted determinand is the suspended solids which is highest for high flow conditions as would be expected but higher at Boddington Bridge than Withy Bridge in mean terms which probably reflects the constant dominant effect of the sewage effluent discharge from the Hayden water reclamation works.

Comparison of these figures for enzyme activity with figures in the literature for the same enzymes is not straightforward because of the different expressions for enzyme activity in use. Some authors for example quote a percentage change in optical density as a substrate such as casein or a fluorescent derivative of a carbohydrate is hydrolysed (Lenhard, 1967; Teuber and Brodisch 1977). Others report numbers of organisms detected as possessing hydrolytic activity using selective media (Hankin and Sands, 1974).

Results quoted by Verstrate et al. (1976) for saccharase or invertase as they termed it taking into account the sample volume were between 40 and 600 mg reducing sugar released/ml sample/hour for a polluted canal and this is in reasonable accord with the figures for Boddington Bridge.

Direct comparison of casein hydrolysis rates in aquatic environments is difficult because of the paucity of published figures. Rawlings and Wood (1978) studied the enzymology of activated sludge systems treating fellmongery waste but their results for non specific protease using an Azocoll substrate were 2-3 orders greater than the figures for Boddington Bridge; this may be a failure to correctly convert experimental conditions for comparison.

Results for soil casein hydrolysis reported by Nannipieri et al (1980) when converted for experimental conditions give an activity per gram of dry soil about 100 times that of the river at Boddington Bridge which might be expected as soils are more concentrated environments.

3.2 Multivariate Data Examination

3.2.1 Scatterplots

Having fitted a reasonable working distribution to the empirical distribution of the raw data it should be possible to take further steps to interpret the attributes under study, in this case enzyme activity, and explain them in terms of other environmental factors. This is in fact the process of statistical model construction. Aquatic environments show high levels of variability and because of that it is difficult to produce completely deterministic models of such water quality characteristics as flow, particulates, organic content or bacteriological quality. A bivariate regression model would usually take the form:

Variable value = systematic component + random component

where the systematic component depends on the value of another variable. This could be expressed as:

$y_r = a + b\,x_r + U_r$ (Cooper and Weekes, 1983)

y_r = dependent variable

x_r = explanatory variable (or independent variable)

u_r = random component values

r = 1 to n

a, b = constants

This would be a linear model although the dependent and explanatory variables may be transformed to achieve an empirical Normal Distribution.

u the random component would be expected to be normally distributed and as a corollary that there should be no correlation between the fitted values and this random component. It is likely in water quality situations, that difficulty would be experienced in controlling that random component without adding so many explanatory variables into a multi-element regression model, that the biological interpretation would run the risk of becoming obscure or trivialised.

A simple and useful first step to find explanatory variables is to draw scatterplots of variables to look for implied relationships. Scatterplots were produced for the two river sites Withy Bridge and Boddington Bridge and also the subsetted data using the MINITAB package. All determinands were included and for selected combinations of determinands log transformations were also plotted. This amounted to a large number of plots that needed to be condensed and summarised. An immediately obvious feature of the bulk of the plots was a lack of clear cut relationships. Many of the plots had points remote from the main group of points. These were probably outliers from the main population of values. Histograms and probability plots already presented illustrate the existence of these extreme values. Techniques for identifying outliers in data sets are not well worked out or easy to apply. Some workers reject values that are more than 2.5 standard deviations from the mean value when the data distribution is approximately symmetrical or 3 standard deviations for skewed

distributions (Cooper and Weekes, 1983). Such techniques of comparing a particular value with a measure of central tendency depend on the model of the underlying distribution in use and the arbitrary removal of one or more individual values will alter the underlying distribution and hence the applicability of the model. In the data being reported the omission of high or low values would run the risk of excluding events with biological significance. Low values may reflect a lack of sensitivity of the analytical techniques being used; for example the saccharase levels in clean rivers often tended to be very low or apparently absent when a numerically low figure was reported as a preference to the more conventional "less than" figure which could not be handled by the statistics package being used. Numerical studies of environmental data such as those by Tyler (1974); Taga and Kobori (1978); and Vives-Rego et al (1986); include the logarithmic transformation of enzyme activity and chemical quality when these are compared. Logarithmic transformation lessens the effect of extreme values. In this present study extreme values are on the whole left in the data sets and explanation is included for their occurrence where possible. Statistics subsequently calculated from such data may be affected disproportionately by extreme values but a false picture of the variability of the natural aquatic environments studied would be given if there were to be anything other than minimal treatment of outliers.

Figures 3.10 to 3.13 illustrate the relationships between enzyme activity and important limnological factors.

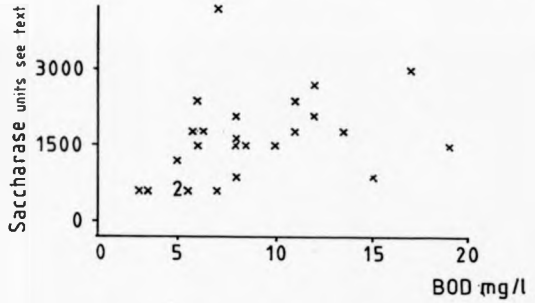
A relationship between saccharase and BOD at Boddington Bridge is shown in Fig. 3.10 to be evident but not clear cut. MINITAB graphical output uses a number to indicate where two data points cannot be separated by the scaling factor used. Transforming both the enzyme activity and BOD to logs hardly improves the visual appearance of the plot as can be seen in the log plot in Fig. 3.10.

Suspended solids and saccharase are related as seen in Fig 3.11 but the influence of the extreme value in the first plot is clear. The second plot which is of the "High Temp" data which does not contain the suspended solids "flier" shows a relatively good linear relationship with saccharase. This would be at low flows and is understandable in that at low flows particles settle out only to be resuspended by modest increase in flows. Scaling of the second plot suggests that there are some high flows in the data set however; these presumably being summer storms.

Figure 3.12 shows the rather tentative connection of temperature and protease activity at Withy Bridge where there is even a suggestion of an initial increase in activity with temperature and a subsequent reduction at higher temperatures. Log transformation hardly improves the plot.

A tentative relationship of protease with reciprocal of flow is discernible in Fig 3.13 which would be interpreted as dilution effect with increasing flow. The second plot of Fig 3.13 shows the reasonable correspondence of log saccharase with log viable count.

Fig. 3·10 Scatterplot Saccharase BOD
Boddington Bridge



[log] Scatterplot Saccharase BOD
Boddington Bridge

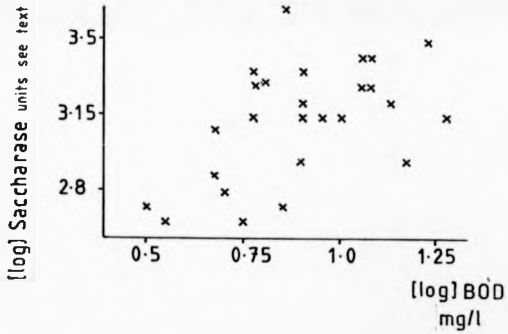


Fig.3.11 Scatterplot Saccharase
Suspended Solids Boddington Bridge

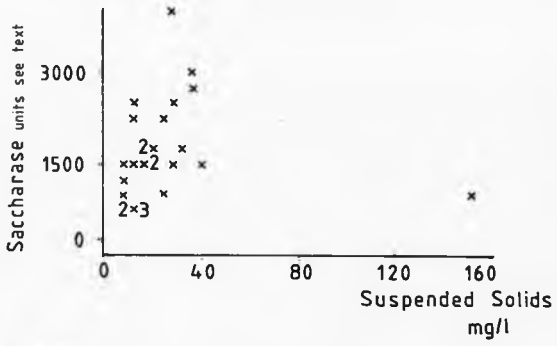


Fig.3.11 Scatterplot Saccharase
Suspended Solids 'High Temp'

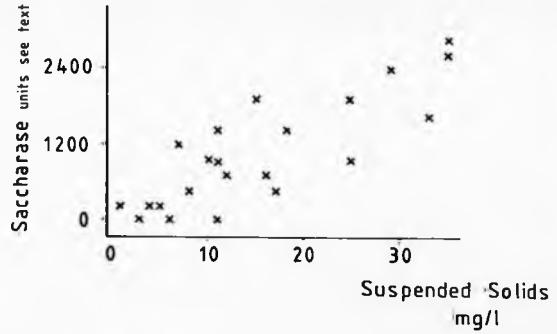
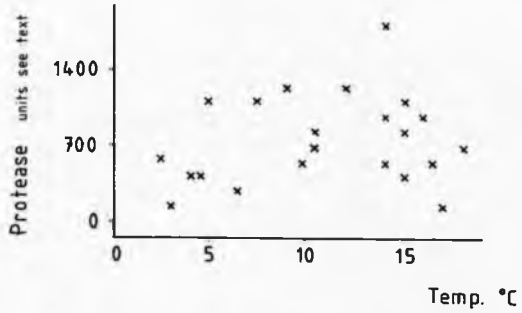


Fig.3·12 Scatterplot Protease Temp.
Withy Bridge



log Scatterplot Protease Temp.
'High Temp'

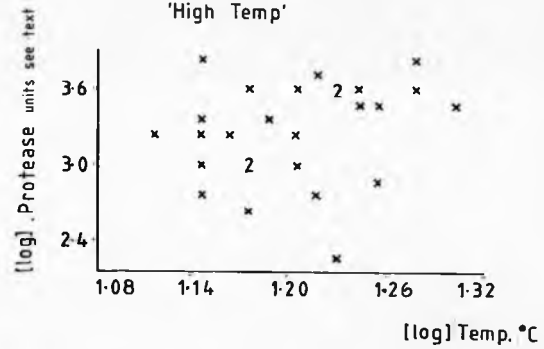
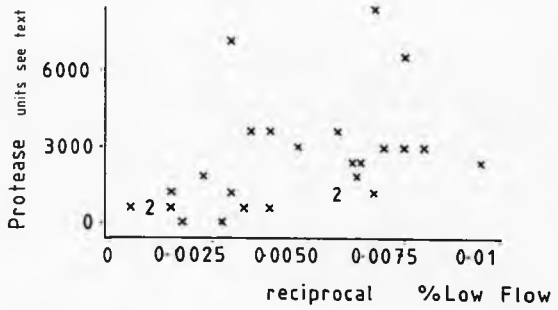
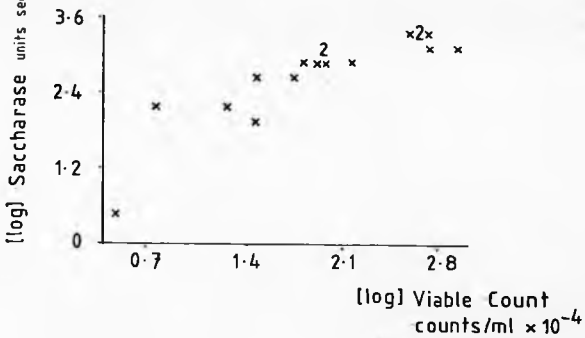


Fig.3·13 Scatterplot Protease Flow 'Low Temp'



log Scatterplot Saccharase Viable Count 'High Temp'



Scatterplots of untransformed and log transformed data suggest that even though patterns are often difficult to see there are few if any genuine non linear relationships. One exception is the relationship of various determinands and flow. They seem to follow a reciprocal relationship. A more speculative pattern for the effect of temperature, when a property such as saccharase activity increases with increase in temperature reaches a plateau then decreases as the temperature continues to rise, would be a polynomial function such as $y = a + bx + cx^2$. The data are too variable to check this proposition and it may in fact be a manifestation of other effects such as changes of flow with season. It appears that log transformation is not strictly necessary to "straighten" a curved relationship but rather is a useful empirical device to modify the effect of extreme values.

Other relationships are suggested by the scatterplots to exist between determinands that have already been shown in Figures 3.10 to 3.13 to relate singly to the saccharase and protease activity.

Figures 3.14 to 3.23 show the apparent multiple correlations of temperature, flow, BOD, suspended solids and viable count.

Figures 3.14, 3.15, 3.16 and 3.17 show the plots of temperature and flow, BOD, suspended solids and viable count. In the case of flow there is an obvious inverse relationship corresponding to the reduction in flow in warm dry summer conditions although there is a spread of flows at the lower temperatures. All of the other determinands show a positive if not very precise link with increasing temperature. There is sufficient variability to make the link between temperature and suspended solids somewhat tenuous.

Fig.3·14 Scatterplot Temp. Flow
Boddington Bridge

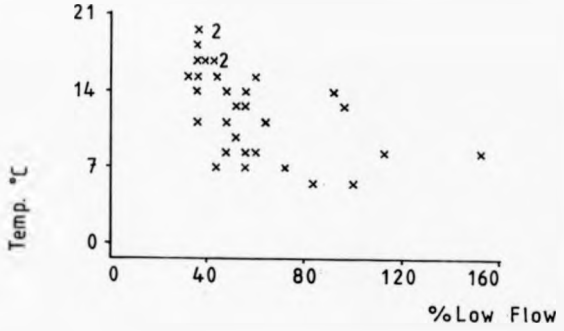


Fig.3·15 Scatterplot Temp. BOD
'High Temp'

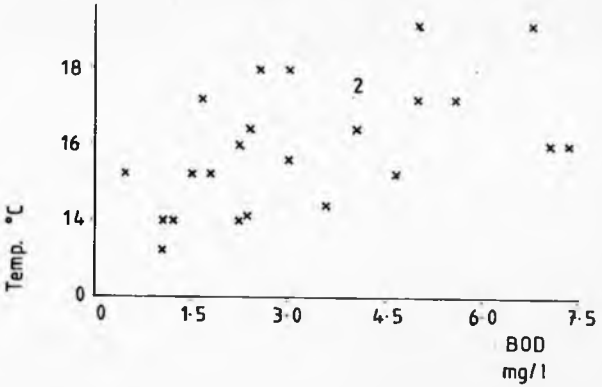


Fig. 3·16 Scatterplot Temp. Suspended Solids 'High Temp'

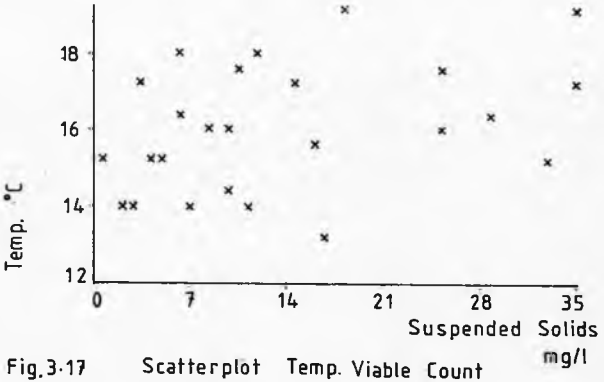
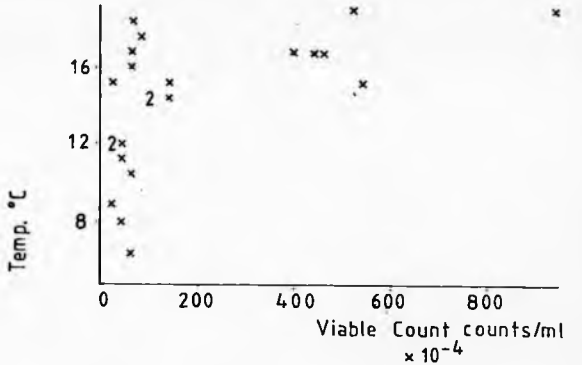


Fig. 3·17 Scatterplot Temp. Viable Count 'Low Flow'



Relationships between flow and BOD, suspended solids and viable count are seen in Figs 3.18 to 3.20 respectively. BOD and viable counts are diluted by rising flows but as might be expected an increase in flow brings particles into suspension as shown at Withy Bridge. In this case the extreme value is not seen because the property plotted is the volatile matter in the particles which was not affected in quite such an extreme fashion as the total solids.

In Fig 3.21 and 3.22 there is an indication of the fairly close link between BOD and solids and viable count. For the plot of BOD and solids a feature noted is the increasing scatter of values as the scale of the determinands increases. This seems a common feature of such environmental data. Fig 3.23 shows a somewhat scattered link between viable count and suspended solids.

Overall these scatterplots illustrate well the difficulty in obtaining an unequivocal picture of the association of environmental properties. The plots shown represented the better examples of a very large number of scatterplots examined. In drawing conclusions from such data it is prudent to always bear in mind this shortcoming in the data.

The method used to measure the suspended solids involves filtration through GFC glass fibre filters. Measurement of enzyme activity and BOD were made on the filtrate produced by this technique. Close relationships exist between the total and filtered figures although filtration tends to spread out the figures perhaps indicating a more consistent level of "soluble" or filterable activity.

Fig. 3·18 Scatterplot Flow BOD
'Mixed'

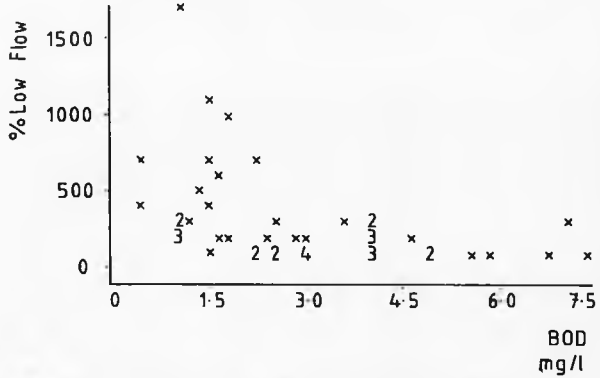


Fig. 3·19 Scatterplot Flow Suspended Solids *
Withy Bridge

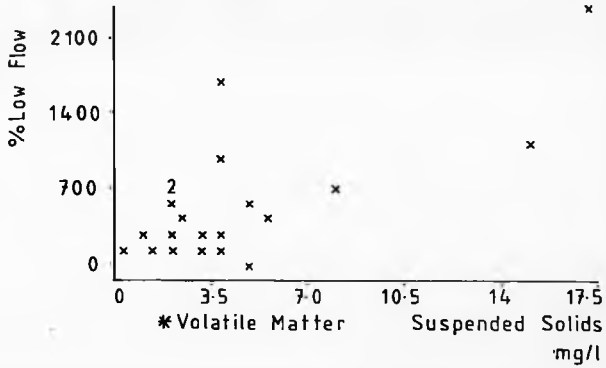


Fig. 3·20 Scatterplot Flow Viable Count 'Mixed'

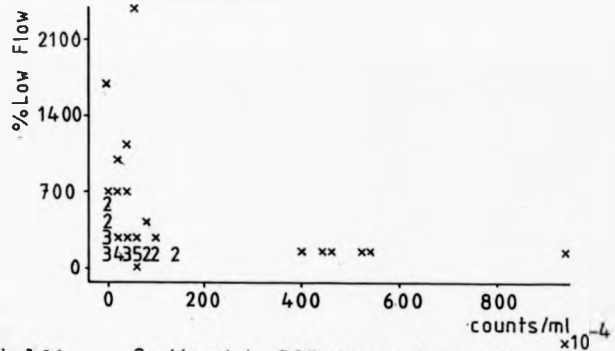


Fig. 3·21 Scatterplot BOD Suspended Solids 'Low Flow'

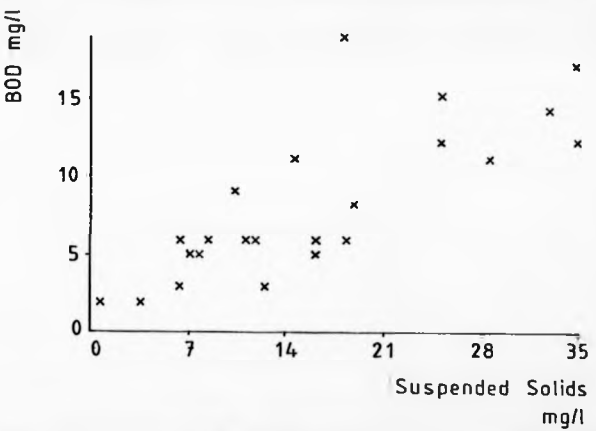


Fig.3.22 Scatterplot BOD Viable Count 'Low Temp'

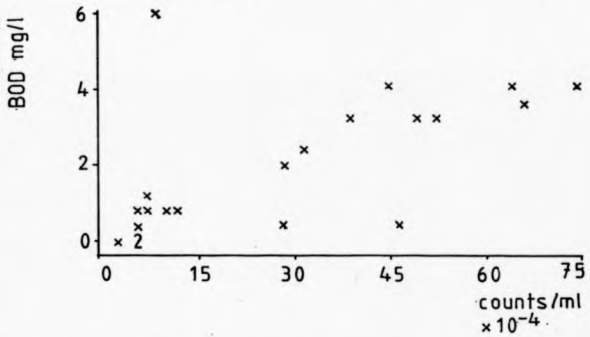
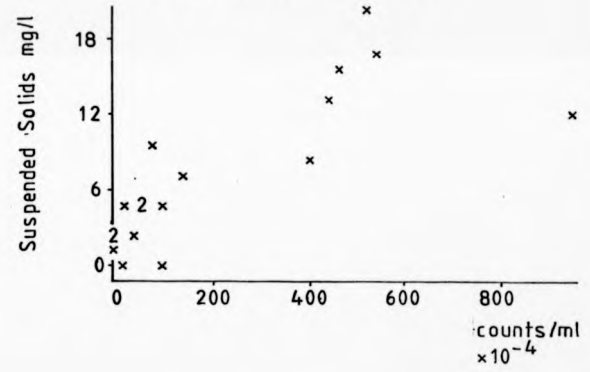


Fig.3.23 Scatterplot Suspended Solids Viable Count 'High Temp'



Because of the pooling and subsequent subsetting of data there are cases where scatterplots reveal the two populations of values. Figure 3.24 shows this where the values of saccharase associated with the dirtier river site tend to occur at higher total oxidised nitrogen concentrations typical of a river with a major input of sewage.

Whilst ammoniacal nitrogen and oxidised nitrogen are measured it is likely that they are closely linked with the presence of treated sewage or other organic pollutants in the water. They would not be expected to directly control measures of heteretrophic activity such as enzyme activity but be coincident indicators of the source of such activity.

3.2.2 Correlation Matrix of Environmental Data

Having carried out a preliminary visual evaluation of the raw data displayed as scatterplots it is clear that relationships are often somewhat tentative. In the absence of obvious relationships it is necessary to determine a statistical measure of association of variables which can then have its significance tested. A relationship of this type which is calculated from the data and which might on testing be acceptable as a non random observation, may nevertheless be difficult to justify in the context of biological interpretation.

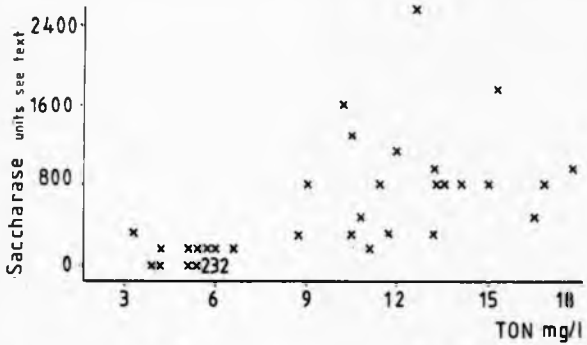


Fig. 3·24 Scatterplot
Saccharase TON
'Mixed'

Fig. 3·25 Histogram
% Saccharase in Particles

Midpoint	Count	
20	1	*
30	6	******
40	7	*******
50	10	**********
60	4	****
70	2	**
80	2	**

Fig. 3·26
Histogram
% Volatile
Matter
in Particles

Midpoint	Count	
0	1	*
10	1	*
20	0	
30	3	***
40	5	*****
50	9	*********
60	5	*****
70	1	*
80	2	**
90	2	**

Boddington Bridge

In order to determine the degree of correlation between saccharase protease and the other environmentally important properties a non parametric rank correlation method was used. This method involves the ranking of both columns of data with subsequent calculation of a conventional Pearson Product Moment correlation coefficient on the columns of ranks. As the method uses ranks of the data there needs to be no assumptions made about the underlying distributions of the data. The significance of the rank correlation coefficient was tested using tables in Bailey 1959.

Tables 3.8 to 3.14 details the relevant correlation information.

Correlations at Withy Bridge are generally not good with only saccharase and total oxidised nitrogen showing some link. Some other determinands demonstrate expected relations for example plate count and BOD.

At Boddington Bridge stronger correlations show up between saccharase and BOD but protease appears to be independent of other factors. BOD associates strongly with suspended solids and plate count. Similarly temperature rise seems to result in an increase in plate count, TON, and chloride. Cause and effect here is difficult to separate as increasing temperature might link through reduced flow and hence increasing proportion of sewage effluent or more intense bacteriological activity in the river.

Table 3.8
Rank Correlation Matrix for WITHY BRIDGE

	Temp	Flow	Saccharase Total	Saccharase Filtered	Protease Total	Protease Filtered	BOD Total	Suspended Solids	Plate Count
Temp			0.191 NS	0.246 NS	0.189 NS	0.224 NS			
Flow	-0.562 **		-0.141 NS	-0.018 NS	-0.054 NS	0.029NS			
BOD Total	0.255 NS	-0.041 NS	0.325 NS	0.276 NS	-0.356 NS	-0.371 NS			
SS	-0.414 NS	0.542 **	-0.194 NS	-0.223 NS	0.029 NS	-0.152 NS	0.276 NS		
Plate Count	0.221 NS	0.042 NS	0.129 NS	0.051 NS	-0.089 NS	-0.248 NS	0.552 **		
TON	-0.222 NS	0.545 *	-0.494 *	-0.330 NS	-0.210 NS	-0.237 NS	0.014 NS	0.285 NS	-0.169 NS
Chloride	0.473 *	-0.368 NS	0.328 NS	0.273 NS	0.314 NS	0.206 NS	0.197 NS	-0.488 *	0.007 NS

* Significant at $P \leq 0.05$
** Significant at $P \leq 0.01$
*** Significant at $P \leq 0.001$
correlation coefficients without asterisks are not significant.

NB FLOW IS STANDARDISED FLOW
NOT RECIPROCAL OF FLOW

Table 3.9
Rank Correlation Matrix for BODDINGTON BRIDGE

	Temp	Flow	Saccharase Total	Saccharase Filtered	Protease Total	Protease Filtered	BOD Total	Suspended Solids	Plate Count
Temp			0.024 NS	0.057 NS	0.180 NS	0.098 NS			
Flow	-0.432 **		-0.248 NS	-0.183 NS	-0.182 NS	-0.292 NS			
BOD Total	0.378 *	0.012 NS	0.519 **	0.412 *	0.391 NS	0.102 NS			
SS	-0.127 NS (-0.026)	0.291 NS (0.214)	0.354 NS (0.431)	0.156 NS (0.189)	0.322 NS (0.301)	-0.247 NS (-0.194)	0.651 *** (0.651)		
Plate Count	0.679 ***	-0.194 NS	0.351 NS	0.261 NS	0.167 NS	0.115 NS	0.694 ***	0.319 (0.331) NS	
TON	0.596 **	-0.410 NS	0.255 NS	0.168 NS	0.161 NS	0.123 NS	0.415 *	-0.017 NS (0.118)	0.417 NS
Chloride	0.606 **	-0.574 **	0.309 NS	0.214 NS	0.100 NS	0.219 NS	0.648 **	0.046 NS (0.214)	0.547*

* Significant at $P \leq 0.05$
** Significant at $P \leq 0.01$
*** Significant at $P \leq 0.001$

figures in brackets in suspended solids row are correlation for data with high suspended solids for sample number 3.81 removed.

correlation coefficients without asterisks are not significant.

Tables 3.10 to 3.14 are all for the combined or subsetted data. In all cases there is a pattern of intensified association between both saccharase and protease with the other limnological factors as well as between the non enzyme activity factors. An almost complete pattern of highly significant correlations is seen for the "Mixed" data. A slightly more selective picture shows up with the "High Temp" and "Low Temp" which are approximately the low flow and high flow subsets of the mixed data. Some of the strong correlations here may well result from the obvious and somewhat artificial two populations of data points seen in some of the scatterplots.

In contrast the "Low Flow" and "High Flow" sets tend to be largely Boddington Bridge and Withy Bridge data respectively and because of the more scant pattern of correlations in the actual river sites the subsets also have fewer links evident.

Overall the enzyme activities show significant links with BOD, particles and bacterial numbers. It appears that total activity generally gives a higher degree of correlation than the filtered activity suggesting that no independent mechanisms are in operation to produce soluble enzyme activity separate from particles.

Table 3.10
Rank Correlation Matrix for 'LOW FLOW'

	Temp	Flow	Saccharase Total	Saccharase Filtered	Protease Total	Protease Filtered	BOD Total	Suspended Solids	Plate Count
Temp			0.080 NS	0.088 NS	0.111 NS	-0.165 NS			
Flow	-0.501 **		0.102 NS	0.121 NS	0.060 NS	0.170 NS			
BOD Total	0.336 NS	-0.033 NS	0.650 **	0.514 **	0.530 **	0.248 NS			
SS	0.117 NS	0.014 NS	0.662 ***	0.404 *	0.449 *	0.076 NS	0.813 ***		
Plate Count	0.608 **	-0.265 NS	0.551 **	0.372 NS	0.557 **	0.180 NS	0.801 ***	0.751 ***	
TON	0.296 NS	-0.178 NS	0.414 NS	0.333 NS	0.409 NS	0.174 NS	0.485 *	0.476 *	0.598 **
Chloride	0.427 NS	-0.368 NS	0.387 NS	0.266 NS	0.463 *	0.305 NS	0.325 NS	0.389 NS	0.759 ***

* Significant at $P < 0.05$
** Significant at $P < 0.01$
*** Significant at $P < 0.001$
correlation coefficients without asterisks are not significant.

Table 3.11
Rank Correlation Matrix for 'HIGH FLOW'

	Temp	Flow	Saccharase Total	Saccharase Filtered	Protease Total	Protease Filtered	BOD Total	Suspended Solids	Plate Count
Temp			-0.013 NS	0.052	0.145 NS	0.252 NS			
Flow	-0.232 NS		-0.425 *	-0.280 NS	-0.422 *	-0.422 *			
BOD Total	0.105 NS	-0.257 NS	0.713 ***	0.700 ***	0.397 *	0.347 NS			
SS	-0.442 *	0.215 NS	0.251 NS	0.167 NS	0.307 NS	0.164 NS	0.470 *		
Plate Count	-0.057 NS	-0.026 NS	0.531 *	0.463 *	0.536**	0.734 ***	0.734 ***	0.711 ***	
TON	-0.049 NS	-0.115 NS	0.445 *	0.498 *	0.511 *	0.414 NS	0.631 **	0.515 **	0.560 **
Chloride	0.227 NS	-0.480 *	0.802 ***	0.741 ***	0.715 ***	0.684 ***	0.687 ***	0.127 NS	0.373 NS

* Significant at $P \leq 0.05$
** Significant at $P \leq 0.01$
*** Significant at $P \leq 0.001$
correlation coefficients without asterisks are not significant.

Table 3.12
Rank Correlation Matrix for 'LOW TEMP'

	Temp	Flow	Saccharase Total	Saccharase Filtered	Protease Total	Protease Filtered	BOD Total	Suspended Solids	Plate Count
Temp			0.112 NS	0.184 NS	0.377 NS	0.509 **			
Flow	-0.377 *		-0.633 ***	-0.554 **	-0.602 **	-0.685 ***			
BOD Total	0.074 NS	-0.537 **	0.787 ***	0.759 ***	0.753 ***	0.652 ***			
SS	-0.268 NS	-0.129 NS	0.156 NS	0.027 NS	0.255 NS	0.122 NS	0.417 *		
Plate Count	0.038 NS	-0.301 NS	0.604 **	0.514 **	0.609 **	0.555 **	0.727 ***	0.604 **	
TON	0.340 NS	-0.550 **	0.787 ***	0.803 ***	0.752 ***	0.649 ***	0.848 ***	0.218 NS	0.498 *
Chloride	0.423 *	-0.664 ***	0.851 ***	0.814 ***	0.840 ***	0.778 ***	0.846 ***	0.146 NS	0.497 *

* Significant at $P \leq 0.05$
** Significant at $P \leq 0.01$
*** Significant at $P \leq 0.001$
correlation coefficients without asterisks are not significant.

Table 3.13
Rank Correlation Matrix for 'HIGH TEMP'

	Temp	Flow	Saccharase Total	Saccharase Filtered	Protease Total	Protease Filtered	BOD Total	Suspended Solids	Plate Count
Temp			0.590 **	0.631 **	0.289 NS	0.123 NS			
Flow	-0.522 **		-0.353 NS	-0.349 NS	-0.321 NS	-0.244 NS			
BOD Total	0.543 **	-0.348 NS	0.854 ***	0.782 ***	0.726 ***	0.505 *			
SS	0.390 *	-0.279 NS	0.782 ***	0.694 ***	0.688 ***	0.399 *	0.890 ***		
Plate Count	0.479 *	-0.366 NS	0.891 ***	0.798 ***	0.786 ***	0.584 **	0.923 ***	0.824 ***	
TON	0.351 NS	-0.175 NS	0.678 **	0.561 *	0.788 ***	0.573 *	0.643 **	0.717 **	0.760 ***
Chloride	0.362 NS	-0.421 NS	0.565 *	0.524 *	0.660 ***	0.639 *	0.507 NS	0.514 NS	0.639 *

* Significant at $P \leq 0.05$
** Significant at $P \leq 0.01$
*** Significant at $P \leq 0.001$
correlation coefficients without asterisks are not significant.

Table 3.14
Rank Correlation Matrix for 'MIXED'

	Temp	Flow	Saccharase Total	Saccharase Filtered	Protease Total	Protease Filtered	BOD Total	Suspended Solids	Plate Count
Temp			0.257 NS	0.257 NS	0.245 NS	0.219 NS			
Flow	-0.622 ***		-0.540 ***	-0.469 ***	0.488 ***	-0.474 ***			
BOD Total	0.390 **	-0.523 ***	0.837 ***	0.796 ***	0.728 ***	0.566 ***			
SS	-0.150 NS	-0.025 NS	0.425 **	0.306 *	0.464 ***	0.250 NS	0.608 ***		
Plate Count	0.511 ***	-0.579 ***	0.688 ***	0.617 ***	0.633 ***	0.541 ***	0.830 ***	0.536 ***	
TON	0.306 *	-0.474 **	0.727 ***	0.670 ***	0.760 ***	0.638 ***	0.748 ***	0.463 **	0.604 ***
Chloride	0.518 ***	-0.699 ***	0.784 ***	0.722 ***	0.783***	0.742 ***	0.795**	0.260 NS	0.645 ***

* Significant at $P \leq 0.05$
** Significant at $P \leq 0.01$
*** Significant at $P \leq 0.001$
correlation coefficients without asterisks are not significant.

3.2.3 Partitioning of Enzyme Activity

As a further way of manipulating the raw data in order to create as good a description of the nature of enzyme activity as possible, the enzyme activity notionally in a particulate form, that is the difference between the total and GFC filtered activity, was expressed as a percentage of the total activity. For comparison the same manipulation was carried out on BOD and suspended solids measurements. In the case of suspended solids the difference between the total and ashed solids was expressed as percentage volatile matter. Figures 3.25 to 3.26 show the typical histograms of these data. It appears that carrying out arithmetic on what in the case of suspended solids data is highly skewed, reduces this skewness to more nearly a normal distribution.

Tables 3.15 lists some basic summaries of the data.

Given that the data on percentage enzyme activities in particulates are quite variable there is some consistency between saccharase at both Withy Bridge and Boddington Bridge. As the two sites are very different in polluting regime the fact of this similarity might suggest a characteristic of saccharase activity on particles that is independent of the level of pollution in the samples. Comparing the two enzymes with equivalent BOD percentages it appears that there is more link between protease and BOD at the two sites as distinct from saccharase and BOD. It could be that saccharase at Withy Bridge is the exception. Volatile matter seems to link in better than particulate BOD with the two enzyme activities.

Table 3.15

Percentage Enzyme Activity, BOD and Volatile Matter in Particles at Various Sites

SITE	SACCHARASE			PROTEASE			BOD			VOLATILE MATTER		
	Mean	95%	C.I.	Mean	95%	C.I.	Mean	95%	C.I.	Mean	95%	C.I.
WITHY BRIDGE	50	36	64	30	20	40	30	22	38	43	32	54
BODDINGTON BR	48	42	53	42	35	48	53	47	59	50	42	57
LOW FLOW	48	42	56	42	35	49	49	41	57	56	49	63
HIGH FLOW	49	38	60	32	24	41	37	29	45	39	30	48
LOW TEMP	48	38	57	38	30	45	39	31	47	44	35	53
HIGH TEMP	50	42	59	37	28	46	46	38	54	50	41	58
MIXED	49	43	55	37	32	43	43	37	48	47	41	53

Rank correlation coefficients were calculated for all sites for combinations of the more important determinands and particulate activity. There seems little general pattern of correlation. At Withy Bridge the only significant correlation was saccharase with plate count ($r = 0.585$ $P \leq 0.01$) which is difficult to explain and might be a fortuitous correlation. At the other sites protease and to a lesser extent saccharase was linked with suspended solids and BOD. This is plausible in that as solids and BOD rise it would be expected that there would be activity associated with what in the case of BOD would be a polluting input. Under these circumstances plate count is also associated with protease. No site shows a significant link between flow and particulate activity although there is a negative correlation with percent volatile matter in the Low Temp data set ($r = -0.412$ $P \leq 0.05$). Only the High Temp data has a link between temperature and activity, protease, ($r = 0.505$ $P \leq 0.01$). High Temp data have correlation coefficients for particulate protease as follows: suspended solids $r = 0.552$ $P \leq 0.01$. At several sites particulate BOD was linked positively with total BOD, total suspended solids and plate count. This is a further demonstration of the inter-relatedness of the different determinands.

3.2.4 Enzyme Activity Expressed in Terms of Other Determinands

A potentially useful way of demonstrating a feature of an attribute in a multifactor data set can be to report a specific activity, specific rate or index number. Goulder, et al (1979); expressed heterotrophic assimilation rate, V max, relative to bacterial population ie V max per bacterium when attempting to recognise pollution stress in estuarine bacteria. Enzyme activity

was plotted against organic matter removal rates for a number of activated sludge plants by Richards et al (1984) and Nannipieri et al (1980), expressed urease and and phosphatase from soils in terms of unit weights of organic nitrogen or carbon when investigating the extraction of enzymes from various soil types.

In this study specific enzyme activities were calculated in terms of enzyme activity per unit BOD, Plate Count and suspended solids. Histograms of these data are markedly skewed being influenced by a relatively small number of very high values. For the Withy Bridge data the single very high value of specific saccharase activity was not associated with a comparable high protease figure and appeared to be caused by a very high total saccharase linked to a low but not exceptionally low plate count. There seems to be some connection between high specific activities of both enzymes and low plate counts but the picture is not very clear. Figure 3.27 and 3.28 illustrate the Histograms of specific saccharase and specific protease for the Withy Bridge site.

Table 3.16 lists summary statistics on specific activites at two sites and the combined data. The gap between the arithmetic mean and the median illustrates the lack of symmetry in the data. A geometric mean may have been a better summary statistic in this case. There is the suggestion of inter-site differences between Withy Bridge and Boddington Bridge with saccharase being slightly higher at Boddington and protease, particularly filtered protease, higher at Withy. It may be on rigorous testing of the data that there is a low level of confidence in that conclusion.

Fig.3-27 Histogram Specific Saccharase activity Withy Bridge

```
Midpoint  Count
       0     11  ***********
      20      5  *****
      40      0
      60      0
      80      1  *
     100      0
     120      0
     140      0
     160      0
     180      0
     200      0
     220      0
     240      1  *
```

Fig.3-28 Histogram Specific Protease activity

```
Midpoint  Count
       0      4  ****
      50      4  ****
     100      5  *****
     150      1  *
     200      1  *
     250      0
     300      0
     350      1  *
     400      1  *
```

Fig.3-29 Scatterplot Viable Count Specific Protease activity Withy Bridge

Table 3.16

Summary Specific Activities Units/Bacteria for Withy Bridge, Boddington Bridge and Mixed

Site	Specific Activity Units/Bacteria											
	Saccharase Total		Saccharase Particles		Saccharase Filtered		Protease Total		Protease Particles		Protease Filtered	
	Mean	Med.	Mean	Med.	Mean	Med.	Mean	Med.	Mean	Med.	Mean	Med
Withy Bridge	25	6	5	2	21	1	107	88	21	12	87	44
Boddington Bridge	26	14	13	5	13	9	80	43	41	13	39	29
Mixed	26	11	9	4	16	6	90	52	33	13	57	32

Med is Median

Specific activities were plotted against BOD for the three data sets and for Withy Bridge; enzyme activity was expressed per unit BOD or suspended solids and plotted against total BOD or total suspended solids respectively.

Figures 3.29 to 3.32

In all cases there appears to be a curved inverse relationship suggesting that for example as BOD increases the activity per unit BOD decreases. This is typical of repression - derepression system with substrate initiating growth but without an equivalent production of activity per cell. Also to be borne in mind in this conclusion is the inter-relations between the more dominant limnological properties already discussed. It could be that the above apparent relation is a minor effect on top of the more major changes in the river when an increase in polluting discharge occurs.

A further manipulation of data from the Withy Bridge samples was to multiply enzyme activities BOD and suspended solids by the appropriate river flow at the time of sampling. These figures for loads were plotted against the range of determinands and rank correlation coefficients were worked out. Scatterplots of load and various determinands showed a variable picture in contrast to the work of Mancsak and Florcsyk (1971). Their work suggested much clearer relationships of flow and quality, to the extent of enabling the typing of watercourses into three categories: Type I heavily polluted, Type II clean rivers and Type II intermediately polluted rivers based on degree of pollution, hydrological characteristics and self purification capacity.

Fig.3·30 Scatterplot BOD Specific Protease activity

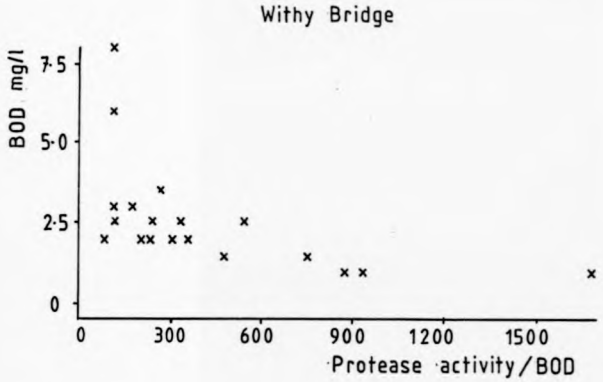


Fig.3·31 Scatterplot Suspended Solids Specific Protease activity Withy Bridge

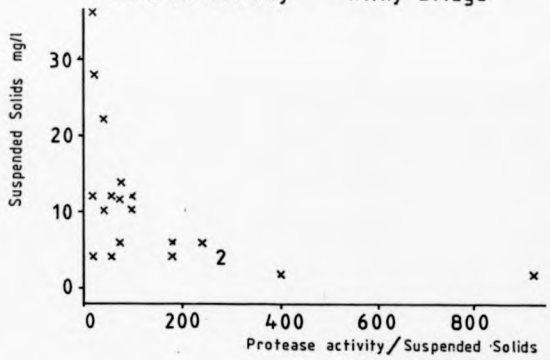
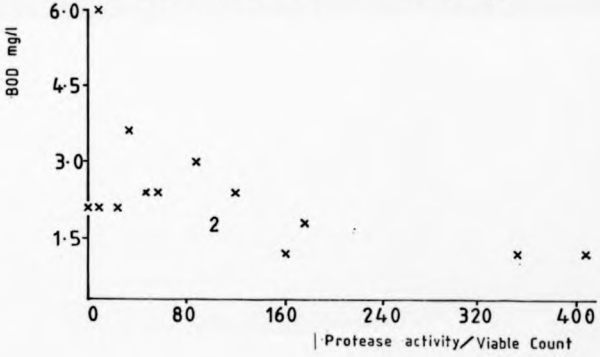


Fig.3·32 Scatterplot BOD Specific Protease activity Withy Bridge



If a characteristic such as saccharase activity were derived from a point source which was roughly constant with regard to its input to the river; a plot of flow against load would be a straight line parallel to the flow axis. Alternatively the activity may have at least in part an origin in the nature of the catchment area such that an increase in flow caused by rainfall would be expected to result in an increase in concentration with increasing flow. Whilst there are not very clear positive correlations for all determinands with flow and in fact several such as chloride show negative correlation with increasing flow, due to the dilution effect of the higher flows, it is likely that attributes such as suspended solids, BOD and enzyme activity would increase as rainfall washed solids off catchments and the higher current velocities resuspended organic sediments. Figures 3.33 to 3.35 suggest this for the Withy Bridge samples. Rank correlation coefficients for flow and saccharase load, protease load, BOD load and suspended solids load are respectively: $r = 0.470$ $P < 0.05$, $r = 0.730$ $P < 0.001$, $r = 0.854$ $P < 0.01$ and $r = 0.891$ $P < 0.001$, confirming the suggestion that these properties tend in part to be generated within the catchment.

Fig.3.33 Scatterplot Flow Protease Load
Withy Bridge

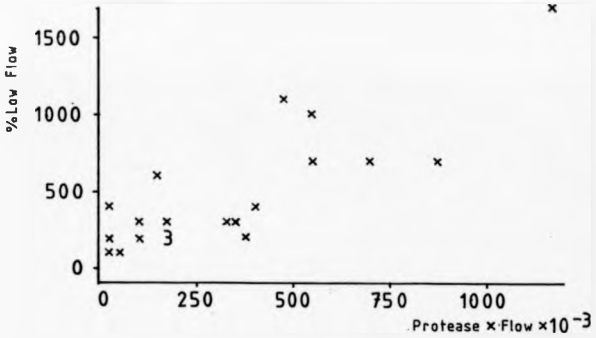
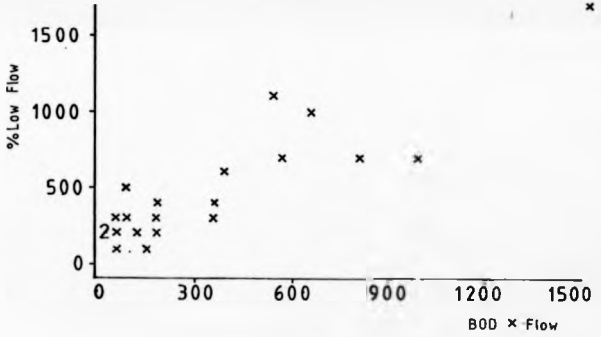


Fig.3.34 Scatterplot Flow BOD Load
Withy Bridge



3.2.5 Association of Enzyme Activity with Different Types of Bacteria

As detailed in the methods section plates used for total viable counting were also counted differentially to distinguish between colony types with the objective of producing counts for the different types of bacteria present in the water samples.

Data from these counts were processed as histograms, scatterplots of counts against other determinands, specific activities in terms of enzyme activity per differential counts and correlations were calculated on ranks of the data as previously. Table 3.16a is a correlation matrix for the differential counts with comparative information for total viable counts for the Mixed data set. It can be seen from Table 3.16a that the Mixed data set and the Allmixed data sets give comparable results for the relationships between total viable count and the major determinands. All differential counts had significant relationships with enzyme activity, BOD and suspended solids with the exception of the correlation of Large Slimy counts with flow and suspended solids. No individual colony type is revealed as having no relationship with enzyme activity or BOD, which might have been the case if for example one of the colony type accurately represented a class of bacteria that were present in the river but had no place in the natural community. Such bacteria could have originated from a point source that discharged in a random fashion, or in a way unconnected with the normal processes of flow and organic input or primary production in the system.

Table 3.16a

Rank Correlation Matrix for Differential Bacterial Counts

	MIXED	ALLMIXED (pooled all data)					
DETERMINAND	TOTAL COUNT	TOTAL COUNT	ORANGE	LARGE SLIMY	ROUND NON CIRCULAR	DENSE WHITE ROUND	TRANSLUCENT
Flow (Standard)	-0.579	-0.521	-0.303 NS	-0.286 NS	-0.250 NS	-0.249 NS	-0.253 NS
Saccharase Total	0.688	0.776	0.754	0.646	0.636	0.680	0.824
Saccharase Filtered	0.617	0.696	0.557	0.673	0.574	0.592	0.659
Protease Total	0.633	0.736	0.792	0.559	0.590	0.674	0.761
Protease Filtered	0.541	0.664	0.586	0.583	0.535	0.517	0.531
BOD	0.830	0.803	0.681	0.562	0.631	0.780	0.861
Suspended Solids	0.536	0.494	0.344	0.320 NS	0.404	0.429	0.590

NS - Not significant at $P \leq 0.05$ all other correlations significant at $P \leq 0.05$

Orange colonies are the most reliably identified types and their counts have a closer link with saccharase and more particularly protease than other types apart from the Translucent types. The Translucent type which tend to be numerically fewer than the Orange type have a stronger association with saccharase and BOD than do the Orange type or the total count. The clearer relationship can be seen also in scatterplots although scatterplots often show bunching of points or the effect of outlying values.

Small data sets for individual sites limits the statistical analysis of differences between sites but counts for the R Chelt at Boddington, Withy Bridge and the River Severn at Tewkesbury show that Orange type predominate with between 40% and 59% of the total count, Translucent types are of a similar order 35-74% with the other types generally 15% or less. A t test on the differences of the mean counts of the Orange types between Boddington and Severn or Withy Bridge showed a non significant difference although sample sizes were very low. The distribution of counts were highly skewed so log transformed data were used for this test.

Whilst this differential counting was subjective, an improvement on simple total plate counting was seen in relation to correlations of bacterial numbers enzyme activities and BOD. Further sampling to build up reliable data at single sites would probably allow improved resolution of the role of bacteria in the production and relationships of enzyme activity with other factors.

3.3 Regression Model of Enzyme Activity

3.3.1 Introduction

Preliminary or exploratory data analysis including modelling of data distributions, production of bivariate scatterplots, calculation of correlation measures and interpretation of relationships based on local knowledge enables a proposed picture to be built up to what controls attributes such as enzyme activity. A more formal way of completing this process is to model the variable in question, for example enzyme activity, so subjecting the model to testing procedures and being able then to use the model predictively for other test situations.

Linear regression models either for dependent and single explanatory variable or with multi explanatory variables are commonly used in many fields. Huttall (1982) derived equations to predict bacterial numbers and heterotrophic potential (V max) in terms of various subsets of determinands such as permanganate value, ammonium nitrogen, chlorophyll a, dissolved oxygen, chloride, flow, suspended solids, temperature, conductivity, pH and total oxidised nitrogen.

A stepwise multiple linear regression analysis was carried out by Brasfield, (1972), to model total bacteria or coliforms or Streptococci in a polluted stream in terms of temperature, pH, detergent, nitrogen, sulphate, chloride, bicarbonate and phosphate. A best fitting line for log Total Bacteria included the three explanatory variables: phosphate, bicarbonate and detergent, each having a positive effect on total bacteria. This

equation would be difficult to interpret without invoking other underlying causes or factors such as polluting inputs. It points out the problems in using regression techniques without some supporting or prompting ideas of what is likely to influence the biology of the habitat being studied.

Examples in the literature of models of enzyme activity in aquatic environments are not as numerous as the more general environmental or flow modelling. Linear relationships were found by Verstraete et al (1976) for phosphatase and numbers of viable micro-organisms and for saccharase after addition of sucrose or peptone to natural waters. Linear regression equations were used by Vives-Rego et al (1986) to study inhibition of biological activities such as glucose metabolism and exoproteolytic activity in seawater. Matavulj and Flint (1987) used a multi element regression model to explain the behaviour of alkaline phosphatase, acid phosphatase, algal activity, viable bacterial count, phosphorus and temperature in a small man made pond. These authors clearly pointed out the problems of inter-relatedness of many physical, chemical and biotic factors in aquatic environments and concluded that multi regression models can often have the number of explanatory variables radically reduced with no serious loss of power to explain and predict dependent variables.

Soil enzymes have in general been more extensively studied than aquatic enzymes and regression models have been used here to relate enzyme activity to our soil characteristics. Verstraete and Voets, (1977) took a range of enzyme activities including saccharase, protease, urease, b glucosidase, phosphatase and

dehydrogenase in addition to microbial counts and Nitrogen mineralisation rates. These biological characteristics were regressed against a wide range of soil factors such as organic nitrogen, inorganic nitrogen, pore volume, drainage characteristics, pH and percent calcium carbonate. Again the number of x variables was reduced from in excess of 12 to a list of 3 that had significant controlling or apparently controlling effects on the y variable.

Regression analysis techniques or the production of linear regression models whether they are for a single explanatory variable or for multiple explanatory variables are mathematically rigorous techniques with procedures for testing the goodness of fit of derived equations. The procedure known as "least squares" is used to calculate a straight line that goes through the data points in such a way as to minimise the squared deviations from the line. Tests for the goodness of fit then tend to concentrate on the differences between the actual values and a fitted value derived from the regression equation and the values of x the explanatory variable.

If regression techniques are to be used for environmental data the technique must be able to handle the inherent problems of such data. These problems include complex or multimodal distributions, presence of extreme values and inter-relatedness of the variables chosen to explain the property under investigation. Strict adherence to the rules of regression analysis requires the explanatory variables to be under the control of the analyst but this is impractical in an environmental study. If it were to be suggested for example that the temperature, BOD, suspended solids

and bacterial quality of a river should be controllable this would clearly not be feasible. Some intuitive assumptions also have to be made about what is likely to be controlling what in a river system. It would be difficult to argue that for example suspended solids controlled temperature or flow in a river but it would not be quite so difficult to argue that organic particulate matter controlled BOD or vice versa or that they were both controlled by some other unmeasurable property such as "catchment" or "input" or "biological state of the river".

Data from the sites at Withy Bridge and Boddington Bridge have been processed by MINITAB to produce regression equations relating saccharase and protease to the other limnological factors. Because of the problems referred to the technique has probably a marginal validity for these data sets but the exercise is justified in that it draws from, and complements the information on rank correlations already presented. Whilst the approach tends to be more descriptive and less quantitative the rigours of the technique highlight the shortcomings in the data and point to further work to improve the basic information.

3.3.2 Regression Equations

Of all the determinands measured a decision was taken to only use temperature, standardised flow, total BOD, suspended solids and plate count as the explanatory variables. Other determinands show good correlations with enzyme activity but not on a general basis and whilst filtered BOD or volatile matter in solids might give a better relation with protease for example at a particular site it

is likely that there is a close link between total and filtered BOD or suspended solids and volatile matter at that site. The decision was made therefore to reduce the amount of computation to a reasonable level. Stepwise regression techniques recommended by Brassfield (1972) to sort out subsets of explanatory variables is available in MINITAB and was used with more or less the full range of determinands. Results were not very helpful in that either one variable swamped all others for example BOD, or groupings emerged; for example particulate BOD with ammonia or conductivity and total oxidised nitrogen which could be considered improbable in that there was no ready biological or limnological justification for their association . This is a further pointer to the inter-relatedness of the data.

Nitrogen either as ammonia or total oxidised nitrogen was left out of the computation as it was considered to be closely linked to BOD or organic pollution. Organically combined nitrogen compounds such as urea or proteins were not measured in this study but might have had significant correlations with enzyme activities.

Sites processed were as already listed for rank correlations with the exception of the Mixed site where it was felt that there was an arbitrary pooling of data. Data for Withy Bridge, Boddington Bridge, and "High Temp" were also regressed as log transformed values with the objective of reducing the effect of outliers. No obviously non-linear relationships had been noted from the scatterplots apart from relationship with flow which tended to be a reciprocal relationship so the flow term was included as a reciprocal which should then have been reduced to a more nearly linear relationship.

Subsets of explanatory variable used to produce equations were as follows:

Temperature

Flow

BOD

Suspended Solids

Plate Count

Temperature	Flow	BOD	Suspended Solids	Plate Count
"	"	"	"	left out
"	"	"	left out	included
"	"	left out	included	"
"	left out	included	"	"
"	included	"	"	"
left out	"	"	"	"
BOD	Suspended Solids	Plate Count		

Production of regression equations with reasonable fits tended to reflect the pattern of rank correlations. Optimum fits were obtained with single predictors although combinations of explanatory variables inevitably increased the R^2 statistic, which expresses the proportion of variability in the dependent variable explained by the chosen x variables. A feature that was evident in the multi-equations was the effect of the x variables on each other in terms of the fluctuating significance of a test statistic calculated from their predictor coefficient divided by its standard deviation. This was noted as different combinations of explanatory variables were used. The test statistic was checked for significance in standard tables of t values (Bailey

1959). This suggests that in a set of 4 or 5 explanatory variables, each of which may on their own give a well fitting equation, only 1 or 2 would be significant. Cooper and Weekes (1983) explain that when two or more explanatory variables are correlated together they are said to be "multi-collinear" and multiple regression analysis suffers from the problem of "multi-collinearity". As a result the precision of regression coefficients is lowered making it difficult to disentangle the marginal effects of individual explanatory variables on the dependent variable. No easy solution to this problem exists although attempts could be made to aggregate explanatory variables. In the case of river data it is difficult to formulate an averaging or weighting procedure to encompass the limnological factors. Workers in related areas have used multivariate statistical techniques including principal components analysis to cope with the problems of multicollinearity (Janicki and De Costa, 1979; Morin et al 1979; Joing et al 1979; Ashley and Lloyd, 1978; Vandeginste and Lersel 1978 and Read et al 1977).

Tables 3.17 and 3.22 list all the better fitting equations defining saccharase and protease activity.

Table 3.17

Regression Equations for Saccharase and Protease Activity at Withy Bridge

Activity		Equations	R^2%
Saccharase	=	126 BOD* - 156	77
Saccharase	=	-17T + 18402 FL* + 58 BOD - 6 SS + 4 VC + 114	77
Saccharase	=	- 9T + 23115 FL* + 55 BOD + 1.4 VC - 11	75
Saccharase	=	47 BOD* - 6 SS + 6 VC - 0.5	64
Protease		No fitted equations	
Log Saccharase	=	2 log BOD* + 1.2	16
Log Protease	=	0.4 log T* + 2.4	11

Note

Abbreviations used in the tables 3.17 to 3.22 are BOD biochemical oxygen demand mg/l; T temperature; ^{o} celsius; FL reciprocal of standardised flow; SS suspended solids mg/l; VC bacterial numbers by plate count, counts/ml and an asterisk indicates that the coefficient for the particular determinand has a significant t value at P 0.05. In all cases of the R^2 the coefficient of determination listed, the value is significant at least at P 0.05; in some cases the correlation is much stronger. Units for enzyme activity are saccharase ng reducing sugar released/ml sample/hour protease ng tyrosine released/ml sample/hour.

Table 3.18

Regression Equations for Saccharase and Protease Activity at Boddington Bridge

Activity	Equations	R^2_x
Saccharase	No fitted equations for unlogged data	
Log Saccharase	= 0.71 Log BOD* + 2.5	26
Log Saccharase	= − 0.99 Log T* + 0.31 Log FL + 0.9 Log BOD* + 0.1 Log VC + 3.90	48
Log Saccharase	= − 0.99 Log T* + 0.9 Log BOD* − 0.1 Log SS + 0.2 Log VC + 3.23	46
Protease	− No fitted equations for logged Protease data	

Table 3.19

Regression Equations for Saccharase and Protease Activity in 'Low Flow' Data Set

Activity	Equations	R^2%
Saccharase	= 96 BOD + 644	28
Saccharase	= 56 SS* + 530	46
Saccharase	= 1.7 VC* + 1028	22
Saccharase	= 54T - 16185 FL + 218 BOD* + 41 SS* - 2.8 VC* + 550	64
Saccharase	= 64T + 222 BOD* + 40 SS* - 2.8 VC + 517	67
Saccharase	= 205 BOD* + 45 SS* - 3 VC* - 247	63
Protease	= 166T - 174377 FL + 556 BOD* - 5.7 VC + 4408	26

Table 3.20

Regression Equations for Saccharase and Protease Activity in "High Flow" Data Set

Activity	Equations	R^2%
Saccharase	= 236 BOD* - 355	47
Saccharase	= 17 VC* + 138	23
Saccharase	= - 78 T + 79662 FL + 191 BOD* - 4 SS + 8 VC + 212	46
Saccharase	= - 83T + 217 BOD* - 9 SS + 9 VC + 453	48
Protease	408 BOD* - 31	50
Protease	- 26T + 96332 FL + 641 BOD* + 10 SS - 32 VC - 191	70
Protease	- 43T + 79153 FL + 662 BOD* - 32 VC* + 108	72
Protease	656 BOD* + 11 SS - 31 VC* - 228	73

Table 3.21

Regression Equations for Saccharase and Protease Activity in "Low Temp" Data Set

Activity	Equations	R^2%
Saccharase	= 191818 FL* + 86	23
Saccharase	= 292 BOD* - 371	54
Saccharase	= 24 VC* + 147	33
Saccharase	= - 77T - 310 FL + 224 BOD* - 21 SS + 20 VC* + 358	60
Saccharase	= - 77T + 224 BOD* - 21SS + 20 VC* + 357	63
Saccharase	= 190 BOD* - 7.5 SS + 21 VC* - 373	63
Protease	= 598 BOD* - 256	45
Protease	= 297315 FL + 626 BOD* + 7.2 SS - 23 VC - 877	47
Protease	= 723 BOD* - 29 SS - 8 VC + 115	45

Table 3.22

Regression Equations for Saccharase and Protease Activity in "High Temp" Data Set

Activity	Equations	R^2
Saccharase	= 139 BOD* - 40	63
Saccharase	= 72 SS* - 33	71
Saccharase	= 2.6 VC* + 533	51
Saccharase	= 25T + 11925 FL + 141 BOD + 48 SS* - 1.5 VC - 879	77
Saccharase	= 16869 FL + 151 BOD* + 46 SS* - 1.6 VC - 572	79
Saccharase	= 157 BOD + 46 SS* - 1.6 VC* - 463	80
Log Saccharase	= 1.71 log BOD* + 1.39	66
Log Saccharase	= 1.30 log SS* + 1.38	51
Log Saccharase	= 0.92 log VC* + 0.96	76
Log Saccharase	= - 1.59 log T + 0.72 log FL + 1.25 log BOD* + 0.4 log SS + 4.9	66
Log Saccharase	= 0.18 log BOD + 0.11 log SS + 0.8 log VC* + 0.93	
Protease	= 251 BOD* + 793	42
Protease	= 117 SS* + 982	38
Protease	= 5.1 VC* + 1685	36

All equations with multiple explanatory variables have significant values of R^2 coefficient of determination but no individually significant predictors.

Table 3.22 (continued)

Log Protease	= 0.79 log BOD* + 2.7	45
Log Protease	= 0.7 log SS* + 2.6	46
Log Protease	= 0.4 log VC* + 2.5	46
Log Protease	= - 4.8 log T* + 0.33 log FL + 1.1 log BOD* + 0.07 log VC	57

None of the other equations have individual predictors that are significant.

For Withy Bridge and Boddington Bridge the number of fitted equations is restricted reflecting the lack of correlations as already commented upon. As expected BOD is the most common strong link although flow and temperature also assume an apparent significance in some of the equations. In two of the equations combining flow with other factors relating to saccharase the flow factor appears to be the significant x variable whereas BOD is the only one that has a significant correlation as a single explanatory variable. This is a manifestation of the ill conditioning caused by multi-collinearity.

Other data sets show groupings of significant factors that relate to the significance of the factors as single explanatory variables for both saccharase and protease.

BOD tends to be the most frequently appearing variable followed by viable count with suspended solids less common and temperature and flow hardly playing any part in the equations for the subsetted data. Another problem, whose solution probably rests in the mathematical quirks of these type of data, is seen for the "low flow" set where saccharase has a positive relation with viable count and yet when viable count appears with the other factors its coefficient then has a negative value. A wide range of numerical values of the regression coefficients for the same factor in different equations is further evidence of the complex or perhaps confused picture of the link between saccharase or protease with the other factors.

3.3.3 Testing of Regression Equations on Other Data Sets

Of the authors cited who produced regression models of environmental data only Matavulj and Flint (1987) carried the testing of the model to the stage of predicting enzyme activity, in their case alkaline and acid phosphatase, for a new set of values of explanatory variables; ending the process by testing the significance of observed and expected activity values. They achieved acceptable levels of agreement for a number of the predictive equations but noted the problem of multi-collinearity, which they termed inter-relatedness, and the potentially profound effect of extreme values in the raw data.

Whilst it is relatively easy to produce regression equations with acceptable fit to a data set from a single site, supporting evidence of a more important general property of any enzyme activity would be provided if the regression equations could be used to predict activity at other dissimilar sites.

Although Matavulj and Flint (1987) used Chi square testing procedures this is more usually applied to frequency data and is used to test the fit of models for data distributions. Little help in formulating a suitable test for predicted activities was forthcoming from the statistics texts consulted (Cooper and Weekes 1983 and Bailey 1959). It was decided to compare the means of the predicted and observed activities using a t test, assuming the data were reasonably normally distributed, and their variances using an F test. A more rigorous test was also proposed involving the regressing of the predicted values on the actual values. On the assumption that predicted = actual if the model is completely

accurate, the regression equation: predicted activity = a + b x expected activity should have a = 0 and b = 1 with a correlation coefficient of high significance. Formulae are given in Moore et al (1972), to compare the slope of the regression line b with a standard k which in this case would be 1. A t statistic is calculated as follows:

$$t = \frac{b - k}{sb}$$

where sb is the standard error of b the regression coefficient

the t value is tested for significance in the usual manner.

To test the regression equations pooled data from a number of sites were subsetted and linked to the model sites as follows:

Model Sites	Test Data
Withy Bridge	Clean
Boddington Bridge	Dirty
Low Flow	High Temp
High Flow	Low Temp
Low Temp	Low Temp
High Temp	High Temp

Criteria for subsetting the data were for Clean and Dirty sites a BOD value of less than or greater than 5 mg/l; High Temp or Low Temp, temperatures less than or greater than 15°, which was the median temperature for the pooled test data. These criteria are somewhat arbitrary but temperature was used because actual flow data were scarce in the test data set and it was assumed that at

low temperatures there would be the bulk of the higher flows ie winter flows associated with rainfall. Of course there are high flows associated with rainfall in summer and low flows associated with droughts in winter. Actual sites comprising the "Clean" and "Dirty" sets are listed in table 3.23 and the "Low Temp" and "High Temp" are mixtures of all sites.

Because of the large number of regression equations produced from the model data and the smaller, but still considerable number of equations which gave a reasonable fit to that data the task of checking the success of the equations in predicting enzyme activity at the test data sites would have been lengthy and laborious had not an element of selectivity been introduced. No convenient computer based facilities were provided by MINITAB to sift out potentially significant sets of predictions and this was done manually by comparing visually the sets of predicted versus measured activities. Of the more than 90 sets of data looked at a relatively small number showed a sufficient degree of agreement to warrant further more formal testing for fit by the regression method proposed. Having 90 sets of data introduces the possibility of apparents fit being a result of chance as well. In contrast the tests of variances and means of the predicted and measured data sets tended to show a good measure of agreement. Null hypotheses that either the variances or the means were not significantly different could in no case be rejected at P 0.05. This might suggest that provided the inaccuracies of the equations were not biased in one direction it would be easy to have mean values of a similar order and the same for the variances.

Table 3.23

List of Sampling Sites Making Up "Clean" and "Dirty" Data Sets

Clean	No of Samples	Dirty	No of Samples
R Severn at Tewkesbury	7	Hayden sewage works final effluent	13
R Chelt at Sandford Bridge	12	Hayden sewage works settled effluent	2
R Leadon upstream Ledbury	2	Hayden sewage works filter effluent	2
R Leadon d/s Ledbury sewage works	4	R Chelt at Inchmoor	17
R Avon at Tewkesbury	3	Ledbury sewage works crude sewage	1
R Swilgate at Tewkesbury	1	Ledbury sewage works settled sewage	1
R Frome u/s Creamery discharge	1	Ledbury sewage works filter effluent	3
R Frome d/s Creamery discharge	1	Ledbury sewage works final effluent	4
Ripple Brook at Tewkesbury	1	Tewkesbury sewage wks final effluent	1
		Creamery discharge biologically treated	
		Longford sewage works settled sewage	2
		Longford sewage works final effluent	2

Predictions from Withy Bridge equations provided no significant correlations with measured activities for the "Clean" data set. It was decided to attempt, in the absence of a set of test data from the Withy Bridge site itself, to choose the nearest equivalent which was judged to be the R Chelt at Sandford Mill Road. Unfortunately no reliable flow data were available for Sandford Mill Road so multiple regression equations including a flow term could not be tested. A set of predictions using the equation linking saccharase with BOD were tested and found to have very poor correlation with r = 0.16 (14 sets of readings) the slope of the regression line of 0.13 which is far removed from the required value of 1.

It was concluded that prediction was unreliable for this site.

A similar picture emerged for the Boddington Bridge equations which failed to predict with any accuracy the activities at the "Dirty" sites. Again a more refined test data set was taken comprising the R Chelt at Inchmoor Bridge which is downstream of Boddington Bridge and the Hayden sewage works effluent which is a major influence on the R Chelt at Boddington.

The equation relating log saccharase to log BOD just failed to produce a significant prediction whereas the following equation: log saccharase = – log TEMP + 0.3 log FLOW + 0.9 log BOD + 0.1 log PLATE COUNT + 3.9 produced a reasonable correlation of predicted and actual with r = 0.71 and a slope of 0.59 which on testing against a standard of 1 gave a t value of 1.9 which was not significant at P 0.05. An equivalent significance test on the intercept of the regression of predicted on measured gave a t

value of 2.73 for the null hypothesis that the intercept was not significantly different from zero. This suggests that there is a small threshold value for the predicted at zero level of measured. Figure 3.36, shows a plot of predicted and measured activity.

The only other data giving any agreement between predicted and measured was the "HIGH TEMP" set. An equation below linked protease activity to temperature, flow, BOD and viable count: Protease = - 469 TEMP + 56708 FLOW + 443 BOD - 1.54 PLATE COUNT + 6853. R^2 for this equation was 40% which is by no means the best fit for Protease in the "HIGH TEMP" data. Figure 3.37 gives the plot of predicted and observed. Correlation of the predicted and observed was significant with r = 0.91 (13 readings) the slope was not significantly different from 1 (t = 0.67) but again there was a value for the intercept.

Finally two equations gave acceptable predictions for saccharase activity: LOG SACCHARASE = 0.9 LOG PLATE COUNT + 1.

Figure 3.38 gives the predicted and observed data. The correlation here was r = 0.89 (32 readings) slope 0.9 (t = 1.2 not significant at P 0.05).

LOG SACCHARASE = 0.2 log BOD + 0.1 log SUSPENDED SOLIDS + 0.8 log PLATE COUNT + 0.9

Figure 3.39 gives the predicted and observed data.

Fig.3·35 Scatterplot Flow Volatile Matter Load
Withy Bridge

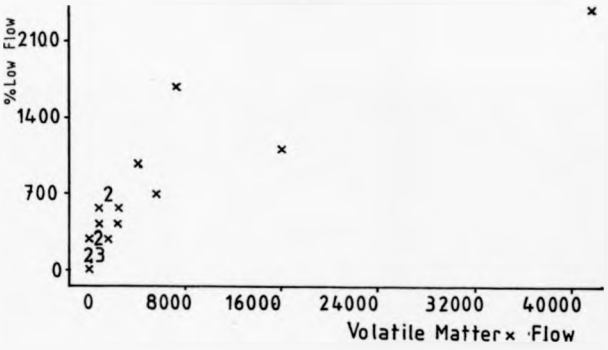


Fig.3·36a Plot Predicted v Observed
log Saccharase activity
Boddington Bridge

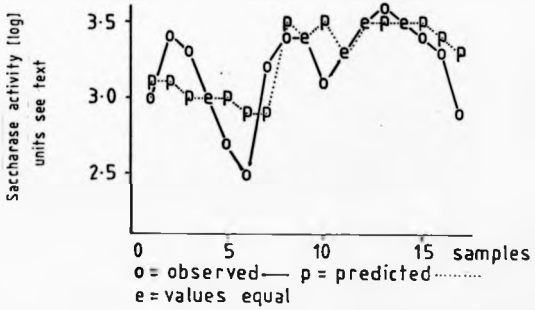


Fig.3·36b Plot Predicted v Observed
Saccharase activity
Boddington Bridge

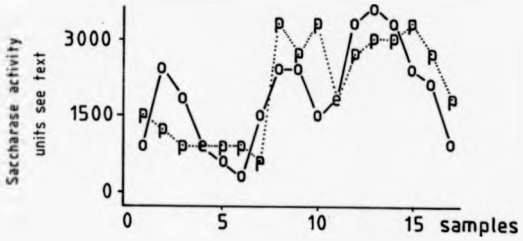
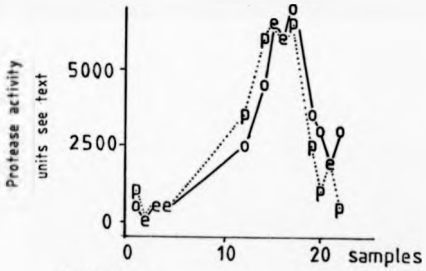


Fig.3·37 Plot Predicted v Observed
Protease activity
'High Temp'



both figs.
o= observed— p= predicted·····
e= values equal

Fig.3·38 Plot Predicted v Observed
log Saccharase activity
'High Temp'

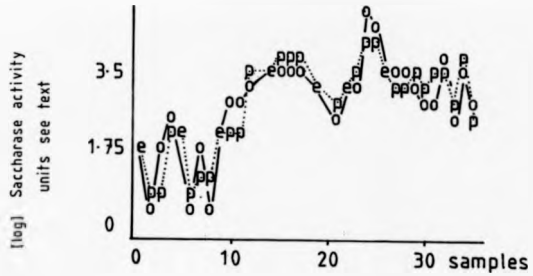
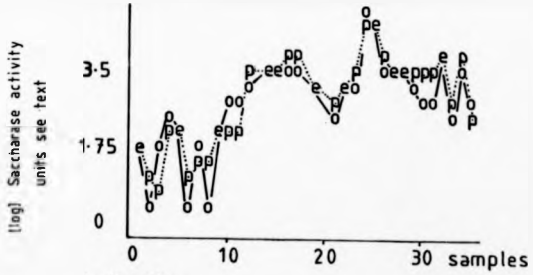


Fig.3·39 Plot Predicted v Observed
log Saccharase activity
'High Temp'



both figs.
o= observed—— p= predicted .....
e = values equal

Correlation here was good at r = 0.92 (32 readings) slope 0.93 which was not significantly different from the ideal of 1 (t = 1.02 not significant at $P \lesssim 0.05$).

3.3.4 Summary

Overall the exercise of producing regression equations for enzyme activity in terms of other familiar and relevant limnological and physiochemical factors has proved useful in helping to establish enzyme activity as a limnological property alongside BOD heterotrophic potential, bacterial biomass and particulate matter. Clearly the choice of explanatory variables is important and it has proved very difficult to choose factors that are independent. Because of the practical limitations of measurement, commonplace factors have been chosen. This was also done in the hope that any explanations would have a ready fit to the pollution control context which was to be partly in the background of the study.

Serious problems of inter-relatedness of factors became obvious and regression equations have suffered in their utility as a result. Nevertheless it has been possible to formulate equations which can explain a large proportion of the variability of enzyme activity in terms of BOD, plate count, suspended solids, and to a lesser extent flow and temperature.

Single sites, particularly clean ones such as Withy Bridge, are relatively difficult to model and this may be due to poor sensitivity in the assays or the disproportionate effects of, for example transient high bacterial counts in a normally low

background situation. It may be that individual sites require individual models. Such site models would allow predictions at that site and comparison between different site models should indicate how changes in explanatory variables relate to changes in enzyme activity. For example it may be that BOD is a controlling factor at a polluted river site where organic effluents dominate as compared to an oligo or mesotrophic lake where phytoplankton activity is the important factor.

For the Water Authority the greater utility would be derived from a universal model for all sites or a manageable number of models for restricted range of sites such as clean rivers, final effluents, polluted rivers as opposed to a multiplicity of individual models.

Pooling of data seems to allow a generalisation of modelling but the reliability of prediction at other test sites is not particularly good. A problem already discussed; that of the basic high variability of the data per se would only detract from the fits of any models. A solution to this problem would be to be very restrictive as to environmental conditions of sampling; for example by sampling only at precise temperatures or flow rates.

Problems of inter-relatedness in such things as BOD, viable count, particulate matter, dissolved solids, nitrogen and flow may be tackled using multivariate techniques other than multiple regression. It would be desirable however to analyse the river system to find any independent underlying factor that may control BOD, bacterial numbers or biologically active particulates.

Having sampled natural environments and attempted an analysis of the resulting environment data, attempts were made to test further, in the more controlled laboratory situation, some of the implied relationships of enzyme activity to bacterial quality and nutritional status as a counterpart to BOD or organic matter. These results are presented in subsequent chapters.

CHAPTER 4

Properties of Enzyme Activity in Natural Waters and Isolated Organisms

4.1 EFFECT OF ADDED SUBSTRATE ON GROWTH OF BACTERIA AND CHANGES IN ENZYME ACTIVITY IN RIVER WATER

4.1.1 Method

Naturally occurring levels of 'enzyme activity' were found to vary with viable count and other limnological factors in a range of watercourse and sewage effluents. It was decided to complement this work on environments of differing BOD, with experiments to investigate the effect of adding substrate to a naturally unpolluted river in terms of any changes in viable count or enzyme activity. An addition of sucrose or casein would simulate samples with a range of organic matter contents so mimicking waters with differing degrees of pollution.

Samples of the River Chelt at Withy Bridge were used for this experiment which was done in two stages taking one enzyme activity each time; sample number 148/83 for saccharase and 154/83 for protease. Both samples were taken under conditions of low flow and each had low values for enzyme activity, BOD, suspended solids and viable count.

In both cases 200 ml of river water were placed in sterile Erlenmeyer flasks and requisite quantities of sterile 1% sucrose or 1% casein were added to give final concentrations of substrate in the range 0, 0.5, 1, 2, 5 or 10 mg/l for sucrose and 0, 1, 2, 5, 10, 20 mg/l casein. These samples were incubated at 20° in a water bath. Enzyme activity and viable counts were determined at intervals up to maximum incubation time of 96 hours. Data on viable count and enzyme activity are presented in Tables 4.1 to 4.4.

Table 4.1

Variation in Viable Count* in River Water Amended With Added Sucrose
Sample 148/83: R Chelt at Withy Bridge

Concentration Sucrose mg/l	Time of incubation (hours)					
	0	6	24	48	72	96
0	4.6×10^4 (4.6×10^{-4})	7.7×10^4 (1.3×10^{-4})	7.9×10^4 (5.2×10^{-4})	8.5×10^4 (11.9×10^{-4})	4.7×10^4 (9.6×10^{-4})	4.8×10^4 (11×10^{-4})
0.5	4.6×10^4 (4.6×10^{-4})	8.0×10^4 (4.5×10^{-4})	5.6×10^5 (0.2×10^{-4})	1.2×10^5 (4.7×10^{-4})	1.7×10^4 (4.1×10^{-4})	3.3×10^4 (9.1×10^{-4})
1.0	4.6×10^4 (4.6×10^{-4})	7.3×10^4 (0.7×10^{-4})	8.4×10^5 (0.3×10^{-4})	2.7×10^5 (2.9×10^{-4})	1.8×10^4 (25×10^{-4})	1.4×10^4 (21×10^{-4})
2.0	4.6×10^4 (4.6×10^{-4})	5.2×10^4 (3.1×10^{-4})	1.2×10^6 (1×10^{-4})	6.6×10^5 (2.4×10^{-4})	1.7×10^4 (4.1×10^{-4})	2.3×10^4 (19.6×10^{-4})
5.0	4.6×10^4 (4.6×10^{-4})	5.1×10^4 (13.1×10^{-4})	1.5×10^6 (1.6×10^{-4})	2.5×10^6 (0.9×10^{-4})	7.2×10^4 (66×10^{-4})	3.1×10^4 (16.1×10^{-4})
10	4.6×10^4 (4.6×10^{-4})	5.3×10^4 (9.8×10^{-4})	1.1×10^6 (2.1×10^{-4})	7.7×10^6 (0.7×10^{-4})	9×10^4 (44×10^{-4})	1.5×10^4 (43×10^{-4})

* Units counts/ml

figures in brackets are specific activity = $\frac{\text{enzyme activity}}{\text{viable count}}$

Table 4.2

Variation in Saccharase Activity Protease in River Water Amended with Added Sucrose
Sample 148/83: R Chelt at Withy Bridge

Concentration Sucrose mg/l	Time of incubation (hours)					
	0	6	24	48	72	96
0	21	10	41	101	45	55
0.5	21	36	9	57	7	30
1.0	21	5	23	79	45	30
2.0	21	16	114	157	7	45
5.0	21	67	252	228	475	50
10	21	52	220	535	400	65

activity expressed as: ng reducing sugar released/ml sample/hour

Table 4.3

Variation in Viable Count and Specific Activity in River Water Amended with Added Casein
Sample 154/83: R Chelt at Withy Bridge

Concentration Casein mg/l	Time of incubation				
	0	6	24	48	96
0	1.27×10^5 (58×10^{-4})	1.2×10^5 (54×10^{-4})	2×10^5 (25×10^{-4})	2×10^5 (16×10^{-4})	4.2×10^4 (48×10^{-4})
1	1.27×10^5 (58×10^{-4})	1.1×10^5 (65×10^{-4})	2.2×10^6 (4×10^{-4})	3.8×10^5 (20×10^{-4})	6.6×10^4 (56×10^{-4})
2	1.27×10^5 (58×10^{-4})	1.4×10^5 (54×10^{-4})	2.9×10^6 (6×10^{-4})	5.7×10^5 (23×10^{-4})	7.8×10^4 (51×10^{-4})
5	1.27×10^5 (58×10^{-4})	1.2×10^5 (59×10^{-4})	4.3×10^6 (5×10^{-4})	7.2×10^5 (24×10^{-4})	6.2×10^4 (131×10^{-4})
10	1.27×10^5 (58×10^{-4})	1.3×10^5 (57×10^{-4})	5.9×10^6 (7×10^{-4})	2.4×10^6 (11×10^{-4})	2.5×10^5 (36×10^{-4})
20	1.27×10^5 (58×10^{-4})	1.1×10^5 (73×10^{-4})	6.9×10^6 (10×10^{-4})	4.4×10^6 (8×10^{-4})	5.3×10^5 (24×10^{-4})

viable count expressed as CFU/ml
figures in brackets are specific activity = $\frac{\text{enzyme activity}}{\text{viable count}}$

Table 4.4

Variation in Protease Activity in River Water Amended with Added Casein
Sample 154/83: R Chelt at Withy Bridge

Concentration Casein mg/l	Time of incubation				
	0	6	24	48	96
0	751	651	501	320	201
1	(751)	711	864	772	369
2	(751)	758	1868	1320	401
5	(751)	711	2224	1696	817
10	(751)	746	4012	2590	910
20	(751)	805	6652	3530	1260

activity expressed as: ng tyrosine equivalent released/ml sample/hour

4.1.2 Results and Discussion

Figures 4.1 and 4.2 show the effect of added sucrose on the viable count and saccharase activity respectively. Without supplementation by sucrose there is little change in activity or viable count and any difference could be explained by the precision of the procedures including the sampling of what is likely to be a suspension of assemblages of particles and organisms. Addition of any substrate results in a 7 to 20 fold increase in viable count after 24 hours and there appears to be an approximate relationship between amount of sucrose added and the growth resulting. After 48 hours there is a further increase for the samples containing the higher levels of substrate but for the concentrations of 2 mg/l sucrose or less there is a reduction. This reduction continues for the remainder of the incubation period with all the samples having a final viable count of a similar order and at levels at which the experiment started.

Changes in saccharase are more random than the growth in bacterial numbers but again after 24 hours there is a general increase in enzyme activity. Further incubation resulted in increased activity with the higher activities associated with the higher initial sucrose additions. After 48 hours there is a substantial decrease in all samples apart from the two with the higher sucrose content. All samples have a final saccharase activity slightly higher than the initial level.

Fig. 4·1 Variation viable count on incubation of 148/83 with sucrose

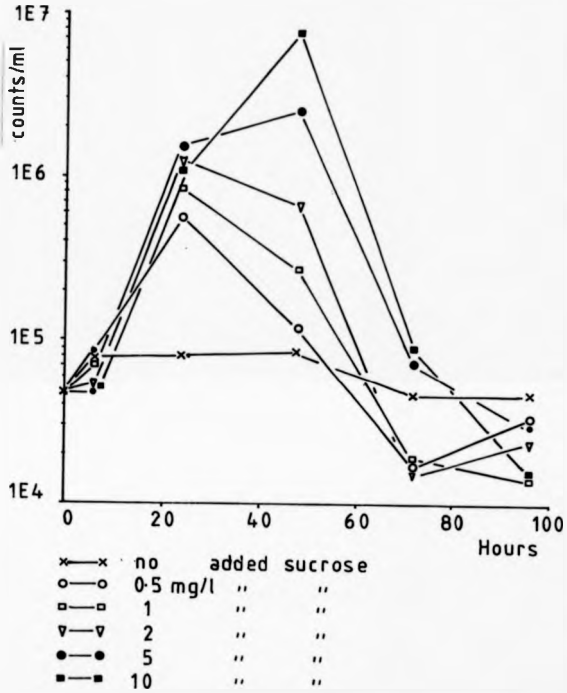


Fig. 4·2 Variation of saccharase activity on incubation of 148/83 with sucrose

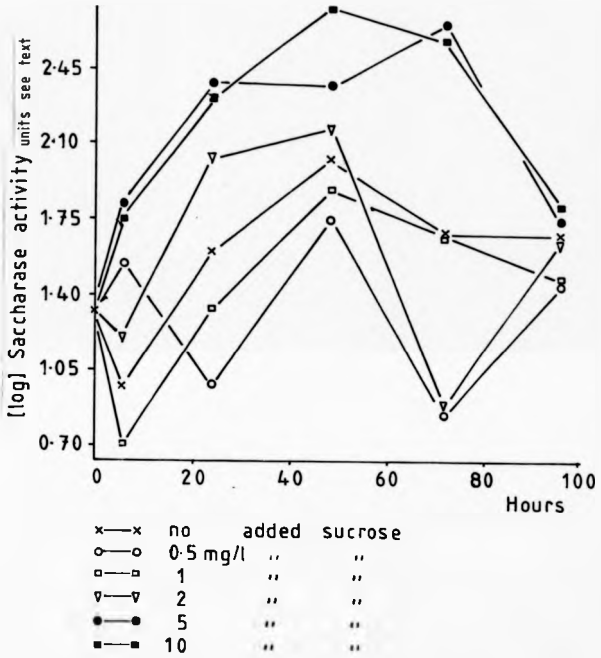


Table 4.1 lists the saccharase activity expressed per viable count and these data qualify the apparent direct link between increasing bacterial numbers and increasing activity. It can be seen that specific activity tends to remain roughly similar for all samples until after 24 hours incubation when there is a significant decrease. This suggests that whilst the addition of substrate stimulates the multiplication of organisms and the production of enzyme activity the amount of enzyme produced per cell is much reduced. An alternative explanation could be that the numbers of non-enzyme producing bacteria increase more than the producers. Without counting the different types of bacteria it is not possible to decide between these explanations.

Figures 4.3 and 4.4 present data for response of viable count and protease in a further sample of river water supplemented by the addition of casein as a substrate. It appears that the addition of casein has an effect on viable count, protease and specific enzyme activity analogous to that of sucrose amendment. If anything the effect of differing concentrations of casein is seen to give a more obviously equivalent change in both viable count and protease activity. These differences persist in the sample after incubation for 96 hours. The sample of river water without added casein has a protease activity which appears to decline in a regular logarithmic fashion whilst there is no corresponding reduction in viable count. This might suggest that enzyme activity is disassociated from the actual organisms and has a half life of its own with maintenance of ambient protease activity somehow residing in the river system from whence the sample was obtained. Perhaps the activity <u>in situ</u> is produced from the sediments and attached bacterial films.

Fig. 4·3 Variation of Viable Count on incubation of 154/83 with casein

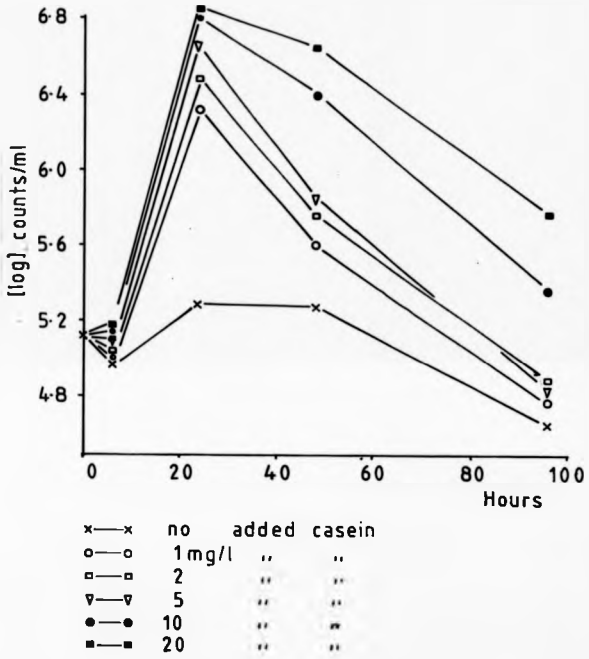
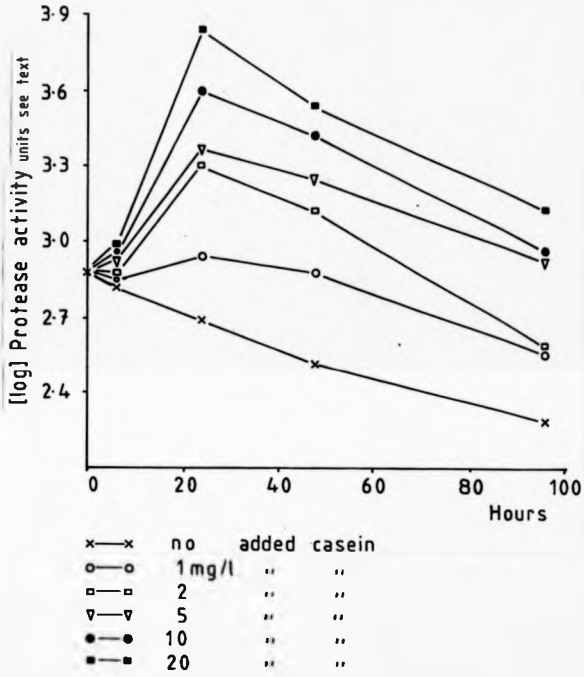


Fig. 4·4 Variation of protease activity on incubation of 154/83 with casein



An attempt was made to look for changes in the bacterial microflora of river water after incubation in the presence of sucrose. This was carried out by categorising the visible appearance of the colonies on the viable count plates. Of necessity the categories were very subjective and any conclusions are therefore approximate. Table 4.5 presents these data expressed as percentages of total count for each category. It can be seen that there is a reduction in the proportion of orange yellow pigmented bacteria and an increase in both white smooth and small colonies. Any differences noted here would probably not be great enough to explain the large changes in specific activity noted in the experiment. Also it seems unlikely that any one group is the main producer of enzyme activity.

An explanation of the substantial reduction in bacterial numbers during the incubation was found when samples were examined at different times under a medium power phase contrast microscope. At 24 hours samples with 2 mg/l or more of sucrose showed noticeable turbidity. The 10 mg/l sample had many bacteria per field; some single rods, couples, short filaments or cocci and some motile bacteria. Very few recognisable cells were seen in the unsupplemented sample which is understandable considering its low count. After 72 hour incubation the picture had altered radically with virtually no bacteria evident but relatively large numbers of what appeared to be flagellated protozoa of the Euglena type. Clearly the stimulation of the bacterial population by the addition of a substrate had led to an increase in predators as would be expected in a natural sample. The decline of the bacterial population and simultaneous reduction in enzyme activity could therefore be explained if part of the activity was closely

Table 4.5

Differential Colony Counts of River Water Supplemented with Sucrose Incubated at 20°C

Colony Type	Initial Count	Sucrose Concentration mg/l					
		0	0.5	1	2	5	10
Orange/ Yellow	25	8	8	7	9	6	1
White Slimy	6	1	1	1	3	1	1
White Smooth	22	21	17	35	29	34	56
White Crinkly	1	1	1	1	1	1	1
Small 0.5 mm dia	37	65	72	56	58	57	38
Spreaders	10	5	1	1	1	1	6

Counts are values expressed as % of total colony counts after incubation for 96 hr at 20°C.

associated with the bacterial cells which had been consumed by the predators. This would also go some way to explaining the changes in specific activity towards the end of the incubation period. A similar picture was seen in the protease run.

4.2 ISOLATION FROM NATURAL WATERS OF ORGANISMS PRODUCING ENZYME ACTIVITY

4.2.1 Methods

Experiments described in Section 4.1 provided strong circumstantial evidence linking increased enzyme activity with the increased bacterial numbers brought about by the addition of the substrates, sucrose and casein, to river water. This growth experiment also suggested that the bacterial microflora might be altered by the artificial stimulation of microbial growth. As an extension of this investigation it was decided to isolate and develop pure cultures of some of the bacterial types commonly seen to be present in natural waters.

Samples 1983/85 and 1984/85 were from the River Chelt at Withy Bridge and Boddington Bridge respectively. They were taken as part of the main survey of enzyme activity in natural waters and one of the factors measured was the plate count of the samples on a casitone-glycerol-yeast extract agar medium. After the plates had been counted a number of colonies were picked off and used to inoculate tubes containing 10 ml of a casitone glycerol yeast extract broth. In order to ensure as near a random selection of colonies for isolation as possible a numbered 1 cm square grid was placed on each plate and the random numbers from a pocket calculator used to pick out squares from which a colony was chosen.

Broth cultures were incubated at 20° for 5 days on a shaking water bath although growth (increased turbidity) was noted in most of the tubes within 2 days.

Growth of the cultures, which were labelled C1 to C15 and D1 to D16, was recorded by measuring their optical density at 420 mm on a spectrophotometer. It was noted that some of the tubes had clumped cultures and others showed more surface growth than growth in the bulk of the broth therefore results of optical density measurements were likely to be variable and this exercise would have to be viewed as a preliminary screening procedure only.

Saccharase and Protease activities of the broth cultures were measured using 0.5 ml of the culture as the sample, with incubation at 35° for up to 19 hours.

Further broth cultures of these isolates were set up, supplementing the broth with sterile sucrose or casein to give a final concentration of supplement of 100 mg/l. This concentration was considered sufficient to be a stimulus to the bacteria to produce enzyme activity without being a major nutritional component of the medium (casitone for example was at a concentration of 5000 mg/l).

Supplemented broth cultures had their growth and enzyme activities measured as described above. For each culture, purity was checked by streaking out on casitone glycerol yeast extract agar plates.

4.2.2 Results and Discussion

Results of these assays are presented in Tables 4.6 and 4.7. The raw data from enzyme assays are absorbance figures which, if actual activities are to be worked out, would be adjusted for blanks, time of incubation and calibration against standard glucose in the case of saccharase, or tyrosine in the case of protease. This processing was not considered necessary for this exercise because simple absorbance figures could be compared providing all other assay conditions were the same. Particular attention was paid to the setting up of a sample blank of the broth culture without the addition of the enzyme substrate. It is likely that the growth of bacteria in a complex medium such as casitone glycerol yeast extract would give rise to the production of reduced compounds or redox conditions which might interfere with the enzyme assays. For comparative purposes and to take account of the likely variability in growth of the cultures the enzyme activities were expressed in terms of their respective culture optical densities and the sample blank was subtracted from this figure to give a corrected specific activity. It was felt that cultures producing activity could be more reliably identified as those showing high specific activity.

The data in Tables 4.6 and 4.7 suggest that cultures vary widely in the extent of their growth under the cultural conditions. A number produce enzyme activity in straight broth eg C6, C12 and D9 which produce both saccharase and protease. Of these C6 also produces a higher level of saccharase activity in the presence of added sucrose and casein. For this culture the addition of sucrose appears to depress the activity of protease. Isolates

Table 4.6

Isolation of Bacteria Producing Enzyme Activity from Sample 183/85
River Chelt at Withy Bridge

Culture	Culture OD 420nm *			Specific Saccharase			Specific Protease		
	CGY Broth	CGY + Sucrose	CGY + Casein	CGY Broth	CGY + Sucrose	CGY + Casein	CGY Broth	CGY + Sucrose	CGY + Casein
C1	0.110	0.048	0.075	0.83	17.06	0.90	0	0.98	2.15
C2	0.075	0.039	0.038	0.72	3.10	2.06	1.24	2.25	5.99
C3	0.135	0.120	0.070	0.55	4.41	2.11	0	0	1.35
C4	0.112	0.105	0.073	0.94	5.64	1.52	0	0	0.14
C5	NO GROWTH		-	-	-	-	-	-	-
C6	0.047	0.122	0.069	2.51	5.38	4.08	2.51	0	0.45
C7	0.067	0.054	0.042	0.56	0.72	1.48	1.17	0.81	3.94
C8	0.093	0.063	0.088	0.23	0.43	0.02	0	0.04	0
C9	0.390	0.305	0.300	0	0	0	0	0	0
C10	0.072	0.045	0.053	0.54	1.10	0.72	1.58	1.00	2.63
C11	0.098	0.068	0.059	0.30	0.74	1.07	0.39	0.47	1.97
C12	0.050	0.048	0.050	1.46	1.10	1.37	2.74	0.98	2.48
C13	NO GROWTH		-	-	-	-	-	-	-
C14	0.077	0.050	0.057	0.26	0.70	0.75	0.70	0.52	1.21
C15	0.064	0.059	0.046	1.12	1.71	1.35	1.71	0.45	3.15

*1 samples for optical density measurement were diluted 4 times.

Table 4.7

Isolation of Bacteria Producing Enzyme Activity from Sample 184/85 River Chelt at Boddington Bridge

Culture	Culture OD 420nm *			Specific Saccharase			Specific Protease		
	CGY Broth	CGY + Sucrose	CGY + Casein	CGY Broth	CGY + Sucrose	CGY + Casein	CGY Broth	CGY + Sucrose	CGY + Casein
D1	0.205	0.158	0.124	0	0	0	1.06	1.17	1.73
D2	0.074	0.065	0.053	0.92	0.96	1.19	1.15	0.98	2.31
D3	0.081	0.077	0.069	0.25	0.53	0.56	0.57	0	1.41
D4	0.072	0.094	0.056	0.23	0	0.76	1.17	0.47	2.76
D5	NO GROWTH		-	-	-	-	-	-	-
D6	0.077	0.096	0.077	0.44	0	0.13	0.22	0	0.08
D7	0.050	0.035	0.048	1.24	3.25	1.21	2.64	3.26	2.20
D8	NO GROWTH		-	-	-	-	-	-	-
D9	0.022	0.092	0.051	4.07**	0.06	1.19	7.13	0.47	1.40
D10	0.075	0.11	0.075	0.46	0	0.39	0.91	0.01	0.39
D11	0.105	0.135	0.126	0.16	1.12	0	0	0	0
D12	0.048	0.065	0.051	1.37	0.49	1.29	2.52	1.04	2.69
D13	0.088	0.087	0.069	0.03	0.03	0.49	2.07	0.14	0.27
D14	NO GROWTH		-	-	-	-	-	-	-
D15	0.118	0.155	0.086	0	0	0	0	0	0
D16	0.380	0.250	0.220	0	0	0	0	0	0

* samples for optical density measurement were diluted 4 times
** specific activity is high because of low growth

C1-C4 have their saccharase activity, and for C2 protease in addition, strongly stimulated by the addition of sucrose or casein.

Culture C9 appears to produce no enzyme activity but this may be a function of the heavy growth as measured by culture optical density. An apparently similar situation is shown for the saccharase of culture D1 where culture OD is high and yet there are positive values for specific protease suggesting perhaps a strongly proteolytic organism.

There appear to be few cultures where protease but not saccharase is detected in the unsupplemented broth but there are several where the addition of casein to the medium stimulates protease production without saccharase production. In general it appears that the presence of sucrose and casein causes an increase in the activity of their respective hydrolytic enzymes.

These experiments indicate that enzyme activity is produced by some but not all of the cultures and that the presence of added substrate stimulate enzyme production in some cases. A further stage is to be described where the nutritional status of the growth medium is more closely specified in order to measure the effect of changes on the production of enzyme activity.

4.3 EFFECT OF COMPOSITION OF GROWTH MEDIUM ON ENZYME ACTIVITY OF ISOLATED ORGANISMS

4.3.1 Growth of All Isolated Bacteria on Minimal Medium

4.3.1.1 Methods

Experiments described in Section 4.2 suggest that in complex media such as casitone-glycerol-yeast extract broth different organisms produce saccharase and/or protease activity and the supplementation of the medium with the enzymes' substrates may increase the activity and apparently in others suppress it. In order to try to reduce the effects of the complex mixture of carbon and nitrogen sources available to the isolated bacteria a series of trials were carried out to grow the organisms in a minimal medium with different combinations of carbon and nitrogen sources. Growth and enzyme activities were measured.

The first minimal medium tried had the basic composition as described in Chapter 2 with ammonium sulphate as nitrogen source. Carbon was provided by glycerol which was added as a 1.28% W/V solution to give a final concentration in the medium of 0.5 g carbon per litre. Tubes containing 10 ml of this medium were inoculated with bacteria grown on casitone-glycerol-yeast extract agar plates.

Tubes were incubated at 20° for 7 days. Only a minority, 7 of the total of 31, tubes showed any growth. It was then decided to repeat the experiment using glucose as an alternative carbon source at the same final concentration of carbon.

4.3.1.2 Results and Discussion

After incubation at 20°, growth was detected in 15 of the 31 tubes. Table 4.8 lists the optical densities of the cultures at 420nm.

From the results of these growth trials it was decided to select the following isolates for further experiments on alteration of growth medium: C1 (good producer of saccharase); C2 (good producer of saccharase and protease); C4 (good producer of saccharase but non producer of protease); C9 (control ie non producer); D7 (producer of saccharase and protease); D9 (producer of apparent constitutive saccharase and protease) and D15 (control ie non producer). After more trials on growth of these organisms in minimal medium with or without the supplementation of growth factors, provided by an addition of 0.01% yeast extract to the medium, the following were settled upon as showing the more convenient growth characteristics: C1, C2, C4 (with yeast extract) and D15.

4.3.2 Growth and Enzyme Production of Selected Organisms Under Different Nutritional Conditions

4.3.2.1 Methods

To investigate the effect of medium composition on the growth and enzyme production of these organisms a series of cultures were set up in 50 ml Erlenmeyer flasks. Different combinations of nitrogen and carbon sources were employed by using ammonium sulphate, sodium nitrate, glucose, sucrose and casein. Because

Table A.8

Growth of Isolated Bacteria C1 - C15 and D1 - D16 on a Minimal Medium

Culture	O.D. at 420 nm	Culture	O.D. at 420 nm
C1	0.37	D1	0.260
C2	0.185	D2	0.002
C3	0.005	D3	0.003
C4	0.185	D4	0.002
C6	0.008	D6	0.460
C7	0.005	D7	0.048
C8	0.008	D9	0.012*
C9	0.450	D10	0.600
C10	0.330	D11	0.490
C11	0.005	D12	0.002
C12	0.040	D13	0.008
C14	0.004	D15	0.600
C15	0.065	D16	0.410

* This tube was also supplemented with 0.01% yeast extract to try to ensure growth as this organism had shown significant levels of constitutive saccharase and protease in the previous experiments.

of the uncertainty about the composition of casein the exact composition of the media with respect to carbon and nitrogen was not known but an attempt was made to produce final nitrogen and carbon concentrations of 1 g/l and 0.5 g/l respectively. As these were screening experiments where the main objective was to detect gross effects it was considered that the actual concentrations would not be critical.

Inocula for these experiments were 0.1 ml of broth cultures from previous experiments and there was the possibility of the introduction of small quantities of nutrients to the minimal media cultures.

Flasks were incubated at 20° in a shaking water bath agitating at 150 oscillations/minute. Incubation was carried on until growth was evident in the flasks. The aim was to allow the cultures to reach stationary phase. An incubation period of 3 to 4 days was used after which the culture optical density was recorded and the saccharase and protease assays were carried out. Because of the likely interference to the tests that would be expected from the glucose, sucrose and casein added to the cultures a full set of sample blanks were set up. Absorbance readings from the enzyme assays were corrected as follows:

corrected absorbance = test absorbance - (sample blank + substrate blank)

The corrected absorbances were then expressed in terms of the optical density of the cultures to give a specific enzyme activity which could be used for comparative purposes. Whilst the absorbance readings were expressed per ml of sample used and had resulted from a similar incubation time used for enzyme assays for all four cultures tested, the values for specific activity in this series of experiments could not be directly compared with specific activities from other experiments because of the differences in experimental conditions. Tables 4.9 - 4.12 present the results from these trials.

4.3.2.2 Results and Discussion

In each of the 4 cases the maximum growth was produced in the minimal medium with ammonium sulphate and glucose. The addition of sucrose did not enhance growth and in the case of D15 appeared to depress it slightly. Similarly the addition of casein to this basic medium had little effect on growth apart from culture C2 where there was enhancement. Apart from in C4, where yeast extract had been added to supply growth factors, sucrose was unable to act as sole carbon source which suggests that extracellular saccharase activity in these organisms, is not of central importance to their metabolism. In contrast in all cases casein could act as sole carbon source producing equivalent growth with either ammonia or nitrate as nitrogen source in C1 and C2, much restricted growth in D15 and showing a preference for nitrate as N source in C4. Casein could act as sole nitrogen source in all cases and as sole carbon and nitrogen sources, with isolates C2 and D15 but showing limited growth.

Table 4.9

Growth and Enzyme Activity of Isolate C1 in Minimal Medium with Different Carbon and Nitrogen Sources

Flask No	Media Composition					Culture OD	Specific Saccharase Activity	Specific Protease Activity
	NH_4	NO_3	Glu.	Cas.	Suc.			
1	X		X			0.98	0.54	2.12
2	X		X		X	0.89	8.7	2.16
3	X				X	-	-	-
4		X	X			0.66	0.68	0
5		X	X		X	0.52	11.6	0.17
6		X			X	-	-	-
7				X		0.20	0.28	2.16
8	X		X	X		0.20	0.52	2.5
9		X		X		0.23	0.09	2.16
10	X			X		0.24	1.06	2.58
11			X	X		0.87	1.66	1.64
12	X			X	X	0.18	6.11	3.33

X indicates that this medium component present

specific activity expressed as $\frac{\text{enzyme assay absorbance}}{\text{culture OD}}$

Table 4.10

Growth and Enzyme Activity of Isolate C2 in Minimal Medium With Different Carbon and Nitrogen Sources

Flask No	Media Composition					Culture OD	Specific Saccharase Activity	Specific Protease Activity
	NH_4	NO_3	Glu.	Cas.	Suc.			
1	X		X			0.36	0	0.73
2	X		X		X	0.26	0	0
3	X				X	0.02	0	0
4		X	X			0.04	6.41	2.27
5		X	X		X	-	-	-
6		X			X	-	-	-
7				X		0.04	0	0.25
8	X		X	X		0.65	0	0
9		X		X		0.03	0	0
10	X			X		0.05	0	0
11			X	X		0.09	0	0
12	X			X	X	0.61	0	0

X indicates that this medium component present

specific activity expressed as $\frac{\text{enzyme assay absorbance}}{\text{culture OD}}$

Table 4.11

Growth and Enzyme Activity of Isolate C4 in Minimal Medium with Different Carbon and Nitrogen Sources

Flask No	Media Composition					Culture OD	Specific Saccharase Activity	Specific Protease Activity
	NH_4	NO_3	Glu.	Cas.	Suc.			
1	X		X			1.53	1.62	1.08
2	X		X		X	1.03	6.92	0.97
3	X				X	0.11	1.0	2.52
4		X	X			1.03	1.42	2.18
5		X	X		X	0.90	8.64	2.78
6		X			X	0.33	0	2.29
7				X		0.53	2.64	1.81
8	X		X	X		1.14	1.71	0
9		X		X		0.51	1.94	1.67
10	X			X		0.14	1.71	0.66
11			X	X		1.02	1.46	1.72
12	X			X	X	0.36	3.31	0.93

X indicates that this medium component present

specific activity expressed as $\frac{\text{enzyme assay absorbance}}{\text{culture OD}}$

NB medium also supplemented with 0.01% yeast extract

Table 4.12

Growth and Enzyme Activity of Isolate D15 in Minimal Medium with Different Carbon and Nitrogen Sources

Flask No	Media Composition					Culture OD	Specific Saccharase Activity	Specific Protease Activity
	NH_4	NO_3	Glu.	Cas.	Suc.			
1	X		X			0.99	0	0
2	X		X		X	0.61	0	0
3	X				X	0.02	0	0
4		X	X			0.28	0	0
5		X	X		X	0.22	0	0
6		X			X	0.05	0	0
7				X		0.01	0	1.8
8	X		X	X		0.60	0	0
9		X		X		0.01	0	1.4
10	X			X		0.01	0	1.9
11			X	X		0.08	0	0.11
12	X			X	X	0.02	0	0

X indicates that this medium component present

specific activity expressed as $\frac{\text{enzyme assay absorbance}}{\text{culture OD}}$

Enzyme activity observations on these cultures were less easy to interpret however. The organism chosen as control: D15 whilst showing reasonable growth failed to produce significant enzyme activity which was unexpected as some growth was shown in media where casein acted as sole nitrogen and or carbon source. This could be explained by the utilisation of spontaneous lysis products or even carry over of nutrients from the inoculum.

Organism C1 showed an elevated specific saccharase and a positive protease when sucrose was present in the medium. This activity however did not appear to result in greater growth and may suggest that sucrose was in fact being used in preference or in parallel to glucose but that other factors limited overall growth. Adding casein to the medium did not seem to significantly alter protease levels which tended to have a common level. Substituting nitrate for ammonia as N source in C1 resulted in a reduced growth but enhanced specific saccharase and the disappearance of protease activity.

Organism C2 had been shown to be a good producer of both saccharase and protease in broth cultures but in the more simple culture conditions used here, there was much less extensive growth and an apparent absence of enzyme activity apart from in flask 4 where specific activities were high but this was probably an anomaly caused by the low growth when it was suspected that residual glucose in the medium might intefere with the enzyme assays.

For the bacterium C4 a slightly different picture was seen probably due to the presence of yeast extract which tended to boost growth and may have allowed the production of enzymes. In nearly all cases both enzymes were measured and this conflicts with the experiments with broth cultures where no activity was seen unless the substrates were present. In flask 6 sucrose is the sole carbon source and growth is evident but no saccharase activity suggesting a direct absorption and intracellular use of the sucrose.

In both cases where sucrose is added to a medium containing glucose and a nitrogen source high levels of saccharase activity are shown with nitrate being the preferred N source but with overall growth not as high as with the medium not containing sucrose.

It may be that changes in nitrogen and or carbon sources would reveal other effects on enzyme activity but the multifactor nature of these type of growth experiments mean that the above trials are only preliminary and should be used to point out gross effects only. Overall it appears that isolate C1 is the best producer of both enzymes and D15 whilst growing nearly as well in the minimal medium does so without significant production of enzyme activity.

4.4 GROWTH AND ENZYME ACTIVITY OF SELECTED ORGANISMS IN THE PRESENCE OF TOXIC CHEMICALS

4.4.1 Growth and Enzyme Production of Selected Organism in Supplemented Minimal Medium

4.4.1.1 Method

Following on from the growth experiments described in Section 4.3 it was decided to select one organism, showing measurable growth and production of enzyme activity in minimal medium, to investigate further. This investigation was to be in terms of the organism's growth and enzyme production in the presence or absence of a range of toxic chemicals of the sort that may be encountered in natural waters subject to pollution by industrial effluents.

Of the organisms that showed an ability to grow in a minimal medium the isolate labelled C1 was considered to be a reasonable test organism because of its production of both saccharase and protease when growing in the presence of substrates for the enzymes.

Accordingly cultures were set up containing minimal medium with glucose (final concentration 0.25 g C/l), ammonium sulphate, casein (final concentration 0.6 g/l) and sucrose (final concentration 0.6 g/l equivalent to 0.25 g C/l). 100 ml Erlenmeyer flasks were used as culture vessels and were inoculated from a previously set up minimal medium culture. Flasks were incubated at 20° in a shaking water bath. 20°C

was chosen for all the growth experiments as being a temperature within the range of those found in natural waters.

Samples were taken at intervals to measure the optical density of the culture and set up assays for saccharase and protease. In order to minimise the effect of the culture volume change caused by the removal of samples; separate runs were set up for saccharase and protease. A further problem that was becoming apparent whilst measuring saccharase by the assay of reducing sugars and protease by the assay of phenolic amino acids was the effect of the added glucose, sucrose and casein on the blank values at low culture optical densities and the added turbidity caused by bacterial biomass at higher final culture densities. To check on the need for blank deductions aliquots of the cultures were processed through the Nelson-Somogyi and Folin procedures without prior incubation ie they were initial blanks.

4.4.1.2 Results and Discussion

Results of the growth curves are illustrated in Fig 4.5 and 4.6.

Growth and saccharase production show increases after 40 hours and growth continues in an approximately logarithmic phase until 70 hours when it levels off. This is also the time of peak saccharase activity. It can be seen that the highest specific activity occurs at 48 hours which is in the middle of the exponential growth phase and may be explained by the organisms metabolic response to an available nutrient source. Specific activity drops back to initial levels as growth slows down and settles at a lower level than at the start of growth.

Fig. 4·5 Growth & Saccharase production of C1 in supplemented minimal medium

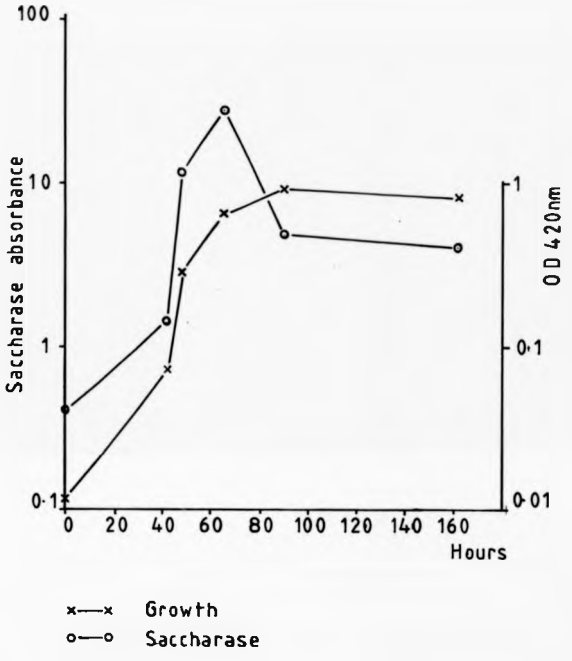
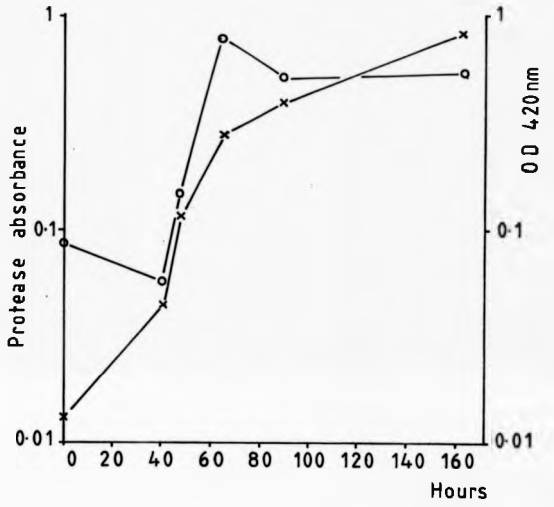


Fig. 4·6 Growth & Protease production of C1 in supplemented minimal medium



Protease production does not follow the same pattern as saccharase production being generally much lower, if a comparison can be drawn on the basis of absorbance readings. As the culture is in exponential growth the specific activity is low and returns to a level in stationary phase which is one half that of the initial level. Culture optical densities for both runs were comparable apart from a shift in the time scale to reach maximum OD.

4.4.2 Growth and Enzyme Production of Selected Organisms in the Presence of Toxic Chemicals

4.4.2.1 Method

To carry out preliminary trials on the effects of some common toxic chemicals on the growth and enzyme activity of isolate 183C1 a further series of cultures with the same basic minimal medium plus carbon and nitrogen sources were set up as for the previous experiment. Table 4.13 gives the details of the toxic chemicals and concentrations used.

Growth and enzyme activities were measured as in the previous experiment. Problems with turbidity were encountered with the gas oil and HCH (hexachlorohexane) flasks where both chemicals are highly insoluble in water. A problem of precipitation of probably zinc phosphate or calcium sulphate was also noticed in flasks 3 and 4. As a consequence of these observations and the fact that the concentrations of toxic chemicals were arbitrary choices based only on notional low or high levels that might

Table 4.13

Toxic Chemicals Used in Growth Trials

with Organism 183C1

Flask	Chemical	Concentration mg/l
1	Gas Oil	1
2	Gas Oil	1000
3	Zinc sulphate	1 as Zn
4	Zinc sulphate	250 as Zn
5	HCH	0.1
6	HCH	10
7	Sodium Chloride (low)	No added chloride control
8	Sodium Chloride (high)	10000 as Cl
9	Potassium cyanide	0.1 as CN
10	Potassium cyanide	10 as CN
11	Phenol	0.1
12	Phenol	10

constitute significant pollution problems if encountered in a natural watercourse with its complex flora and fauna, these experiments were only taken as an indicator of possible effects on growth and enzyme production.

4.4.2.2 Results and Discussion

Table 4.14 gives the results of these trials. Culture growth and enzyme activities are expressed as a percentage of the control culture in flask 7 which has no added chemical.

Concentrations of toxic chemicals were chosen in an arbitrary fashion but it can be seen that all chemicals have measurable effects on growth, activity or both. In all cases apart from the lower level of zinc growth represented by optical density of the cultures is reduced by the toxins and the higher concentration of toxin has the greater effect in reducing growth. Phenol has a marginal extra effect at the higher concentration but cyanide, gas oil and chloride have very significant depressing effects on growth. Zinc appears to cause increased growth but this may be an interfering effect of precipitation of insoluble zinc salts on mixing with the culture medium.

As for the effect of toxins on enzyme activity none of the lower concentrations depress saccharase but protease is completely inhibited by gas oil and significantly inhibited by zinc at lower levels.

Table 4.14

Effect of Toxic Chemicals on Growth and Enzyme Production of Organism C1 in Minimal Medium

Flask	Toxic Chemical	Conc mg/l	Relative Activities		
			Culture OD	Saccharase	Protease
1	Gas Oil	1	61	175	0
2	Gas Oil	1000	16	0	0
3	Zinc	1	138	275	33
4	Zinc	250	13	0	110
5	HCH	0.1	56	100	133
6	HCH	10	31	50	50
7	Chloride	Control	100	100	100
8	Chloride	10000	13	25	23
9	KCN	0.1	88	125	133
10	KCN	10	2	0	33
11	Phenol	0.1	64	150	117
12	Phenol	10	57	125	50

Relative activities are expressed as a % of the control figures

At the higher level of toxin saccharase is inhibited 100% by gas oil, zinc and cyanide. Protease does not follow the same pattern being reduced in activity on average about 70% by the higher levels of added chemical. Saccharase activity reductions caused by increased toxin concentration are comparable in scale to the growth reductions but protease as stated follows a different pattern.

As a further comparative experiment the effect of one toxic chemical on the isolates: C1, C2, C4 and D15 was checked in terms of growth and enzyme activities. The same basic medium was used as in the previous experiments (the medium for organism 183C4 had 0.01% yeast extract added) as was the methods of measuring enzyme activities. Potassium cyanide was chosen as the toxic chemical and three concentrations were used those being 0, 0.25 mg/l and 2.5 mg/l. The results for culture optical density, saccharase and protease were expressed as a percentage of the figure for no cyanide.

Table 4.15 lists these results.

The growth and enzyme activity of different organisms in the presence of two concentrations of potassium cyanide illustrates the general effects of cyanide at the same time as pointing out the differences in the response of the different individuals. The two concentrations of cyanide are different from those used in the experimental results presented in Table 4.15 but are consistent with those concentrations and illustrate the rapid drop in growth caused by slight increases in cyanide concentration. Organisms C2 and C4 show similar effects on

Table 4.15

Effect of Potassium Cyanide on the Growth and Enzyme Production of Organisms C1, C2, C4 and D15 in Minimal Medium

Organism	Cyanide Conc mg/l	Relative Activity % Control		
		Culture OD	Saccharase	Protease
C1	0.25	70	67	182
C1	2.5	4	33	5
C2	0.25	100	16	150
C2	2.5	30	0	0
C4	0.25	96	100	100
C4	2.5	12	0	0
D15	0.25	91	0	0
D15	2.5	90	0	0

Relative activity expressed as % of control figures

growth reductions with the saccharase activity markedly affected by the lower concentration of cyanide. The higher concentration of cyanide causes complete inhibition of saccharase. In no case apart from D15 is protease reduced by the lower concentration of cyanide but is completely inhibited in all cases by the higher concentration.

Organism D15 which acts as a non producer control for enzyme activity shows only marginal reduction in growth for either cyanide concentration.

4.5 PRODUCTION AND SURVEY OF ENZYME EXTRACTS FROM ISOLATED ORGANISMS

4.5.1 Production of Cell Cultures and Crude Enzyme Extracts

4.5.1.1 Method

Studies of enzyme activity in soils and aquatic environments have included results on extracted or purified enzymes when authors wished to investigate the kinetic properties of the enzymes. Jones (1979b) extracted enzymes from lake sediments by ultrasonication in the presence of surfactants. Reichardt (1979) measured dehydrogenase activity in seston and sediments from Lake Constance and cultures of *Cytophaga johnsonae* all after extraction by mild surfactant treatment. The difficulty of separating enzymes and other proteins from soils where soil colloids have a strong sorptive action has been pointed out by Nannipieri (1980), who used sodium pyrophosphate to solubilise the colloids thus releasing enzyme and microrganisms for subsequent extraction. Soil hydrolases were extracted by

Batistic et al (1980) using EDTA. The extracts were fractionated and concentrated by gel and anion-exchange chromatography prior to the estimation of optimum pH and temperatures for activity and Michaelis constants. The 4 organisms used in previous experiments were therefore produced in culture in order to prepare crude enzyme extracts for characterisation.

Cultures were set up in 100 ml Erlenmeyer flasks using minimal medium to which was added glucose, casein, sucrose and in the case of C4 yeast extract as described in Section 4.4.1.1. Inocula were previously subcultured in minimal media with glucose as carbon source. Ammonium sulphate was the nitrogen source in both cases. The flasks were incubated at 20^{o} in a shaking water bath for 4 days when reasonable growth and pigment production in certain cultures was noted. Replicate cultures were processed with subsequent bulking of separated cells.

It was assumed that after 4 days incubation the cultures were at stationary phase and the cells were harvested by centrifugation (20 min at approximately 20,000 g). A quantity of the supernatant was retained and stored at 4^{o} for subsequent assay of its enzyme activity.

Pellets produced by centrifugation were resuspended in 10 ml of cold sterile distilled water in Universal bottles. Cell suspensions were disrupted by ultrasonication in an MSE ultrasonic disintegrator. Each aliquot was given 4 bursts for 45 sec at an amplitude of 10 microns cooling between treatments

in ice and checking progress microscopically. At the end of the treatment few if any intact cells were visible. Portions of the crude extract were stored at $-20^{\circ}C$ until needed.

4.5.1.2 Results of Crude Extract, Enzyme Activity Assay

An initial assay of the saccharase and protease activities of the crude extracts and respective supernatants was carried out in the normal way but incubating at $35^{\circ}C$ for 2.5 hours. Assumptions were made about the required sample sizes and the incubation time and the results are summarised in Table 4.16. The results are expressed as absorbance increases per unit time and unit sample volume in order to compare with the results of assays on activities shown by cell cultures grown under identical conditions and used as the controls in experiments reported in Table 4.15.

In all cases apart from protease in C1 activity is concentrated in the cells and is much greater than in the whole cultures although a concentration of the extract occurred when the cell pellet was resuspended. Overall the activities are not as high as had been expected but there was sufficient increase in absorbance having taken a blank value into account to allow further characterisation of the extracts. Organism D15 was originally included as a non producing control for comparative purposes but it can be seen that whilst whole culture activities are low there is a very significant protease activity and positive saccharase activity seen in the crude extracts. These probably originate from inside the bacterial cells.

Table 4.16

Enzyme Activities for Whole Cultures, Crude Extracts and Cell Suspension Supernatants for Organisms C1, C2, C4 and D15

Organism	Saccharase			Protease		
	Whole Culture	Extract	Supernatent	Whole Culture	Extract	Supernatent
C1	0.08	2.38	0	0.03	0.02	0.07
C2	0.1	0.30	0.04	0.003	0.40	0
C4	0.47	10.4	0.03	0.10	0.38	0.03
D15	0.04	0.72	0.44	0	2.66	0.003

Enzyme activities are expressed as absorbance increase/ml sample/hour.

4.5.2. Activity Profiles for Crude Extracts with Variation in pH, Temperature and Time of Incubation

A series of assays for saccharase and protease were set up using standard conditions described in Chapter 2 to investigate the effect of varying the pH or temperature or incubation time. For comparative purposes the absorbance figures for each set of conditions and for each organism are expressed as proportion of the maximum figures. Table 4.17 and 4.18 list these figures for the pH and temperature profiles of saccharase and protease respectively. Fig 4.7 shows the saccharase activity of C4 and protease activity of C2 with prolonged incubation.

Two of the organisms, C1 and C2 have pH optima for saccharase around pH6 with steep decline on either side of this figure whereas the other organisms saccharase shows a more even spread with peak around pH7. A similar shape of profile is evident for temperature dependence with organism C2 having an apparent almost evenly spread profile. Organism D15 shows less easily explained behaviour in that activity is high at the extremes of temperatures used. This may be explained by the presence of more than one enzyme system in the extract. It is possible that the sensitivity of the assays is not good enough to resolve differences between different pH or temperature conditions and repeat runs with blanks and replicate would be needed. Protease activity is seen to be optimum at 35^{o}C in all cases with clearly defined peaks. Conditions of pH for optimum protease activity are at or around pH7 for organisms C1 and C2 but higher for C4 at pH 9 and much lower at pH 6.5 for D15 which again shows atypical behaviour in that authors have reported pH

Table 4.17

Temperature and pH Profiles of Saccharase Activity for Enzyme Extracts from Isolated Bacteria

Conditions	C1	C2	C4	D15
pH 4	12	84	19	91
6	100	77	100	75
7.5	63	100	33	100
9.1	15	97	9	61
11	12	94	9	72
6°	55	90	74	100
20°	95	100	87	93
35°	100	94	100	85
50°	45	94	42	93
65°	31	94	29	98

Saccharase activity expressed as % of maximum

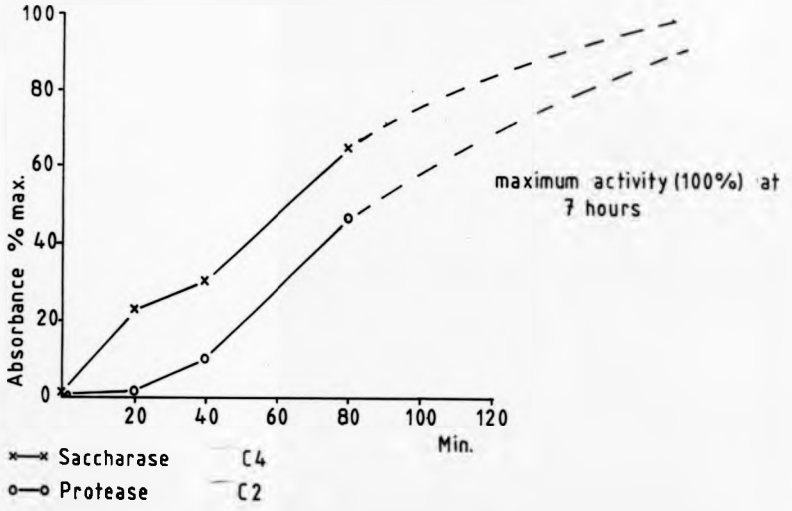
Table 4.18

Temperature and pH Profiles of Protease Activity for Enzyme Extracts from Isolated Bacteria

Conditions	C1	C2	C4	D15
pH 4	36	19	35	80
6	36	94	83	100
7	100	100	95	71
9	43	90	100	57
11	26	77	37	14
6°	45	32	43	53
20°	49	43	79	53
35°	100	100	100	100
50°	43	92	69	58
65°	51	26	59	100

Protease activity expressed as % of maximum

Fig. 4.7 Time course of enzyme reaction in extracts



optima for protease activity generally higher than pH 7 (Lenhard, 1965; Sridhar and Pillai, 1973; and Verstraete et al 1976).

The time of incubation data are inadequate to accurately describe the complete course of the breakdown of the substrates. Over the first 80 minutes of incubation there is an approximately linear increase in production of reducing sugars from sucrose and based on interpolation between that time and a reading at 7 hours which is assumed to be a maximum, it is likely that linear rates of activity continue for up to 3 hours at the substrate concentration used. Protease activity is less clearly linear, at least for the first 20 minutes, but thereafter the limited data suggest a more direct relationship of release of tyrosine from casein with increasing time.

4.5.3 EFFECT ON ENZYME ACTIVITY OF VARIATION IN SUBSTRATE CONCENTRATION AND ADDITION OF TOXIC CHEMICALS

4.5.3.1 Introduction to Kinetic Experiments

To fully characterise enzymes and enable comparison with extracts from other sources it is usual to investigate the kinetics of the catalysed reactions. Michaelis-Menten kinetics are often taken as a suitable model with the Km, the Michaelis constant, and Vmax or maximum velocity of reaction being the defining parameters. Information about the mode of action of enzymes and substances inhibiting their activity can be obtained from measurements of Km and Vmax. Duddridge and Wainwright (1982) used different graphical techniques to plot data on

substrate concentrations and reaction velocities of amylase, cellulase and urease activities in watercourse sediments amended with respective substrates in order to test the Michaelis-Menten model. They concluded that whilst a sensitive technique such as the Eadie-Hofstee plot (Dixon and Webb, 1967) could show up a lack of fit of Michaelis-Menten kinetics. Provided that certain assay conditions such as linearity between activity and substrate concentration, enzyme concentration and length of incubation, were maintained, the Michaelis-Menten model was adequate when studying effects of heavy metals and other xenobiotics on microbial activity in river sediments. Calculation of Km and Vmax for different hydrolytic enzymes in soils has been carried by workers attempting to describe the mode of action of soil enzymes (Batistic, et al. 1980, Pettit, et al 1977). Reichardt, et al (1976) studied the origin and nature of alkaline phosphatase enzymes in lake water using measured Km and Vmax values to compare with previously reported figures for blue green algae from the same lake in order to define the contribution made by phytoplankton to overall phosphatase activity. In a survey of protease activity in sewage, sludges and effluents Sridhar and Pillai, (1973) localised maximum activity in activated sludges which were treated with chloroform to extract protease. This protease was assayed using casein at different concentrations and a Km value was calculated although no comparison was made with enzymes from other sources.

4.5.3.2 Methods

Extract C4 was selected as a good source of saccharase and C2 as a source of protease for assays to check on agreement of Michaelis-Menten kinetics and to calculate apparent Km. Trial runs set up as standard assays with range of subtrate concentrations established the appropriate range of substrate concentration, sample size and incubation time. Substrate concentrations for sucrose ranged from 3.65×10^{-4} M to 3.65×10^{-3} M. For protease assay the nature of casein is such that expression of molarities is difficult so concentrations in mg/l were used and these ranged from 500 to 5000 mg/l. Reaction velocity units do not affect the calculation of Km and tend not to be standardised in studies of aquatic and soil enzymes although authors often express activity as molar units per unit sample volume or unit weight of protein in sample. (Reichardt, et al. 1967). In the current work saccharase activity was expressed as increase in absorbance/0.02 ml sample/hour; protease activity was increase in absorbance/0.3 ml/sample 4 hour. Substrate blanks were set up omitting sample.

4.5.3.3 Results and Discussion

Results were plotted using the Lineweaver-Burk method Fig 4.8 and 4.9 present these plots.

Fitting a least squares line to the plot of saccharase activity in Fig 4.12 gave a Km of 3.2×10^{-3} M. The equivalent figure for protease is a Km of 30 mg/l.

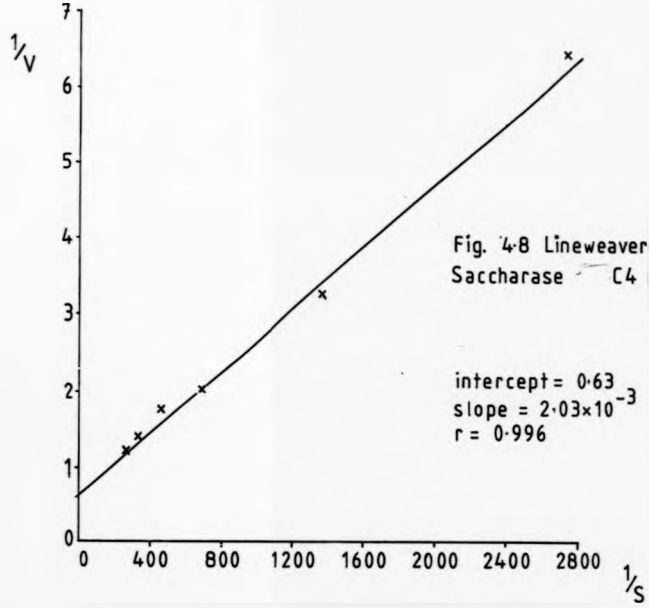


Fig. 4·8 Lineweaver-Burk plot
Saccharase C4 extract

intercept = 0·63
slope = $2 \cdot 03 \times 10^{-3}$
r = 0·996

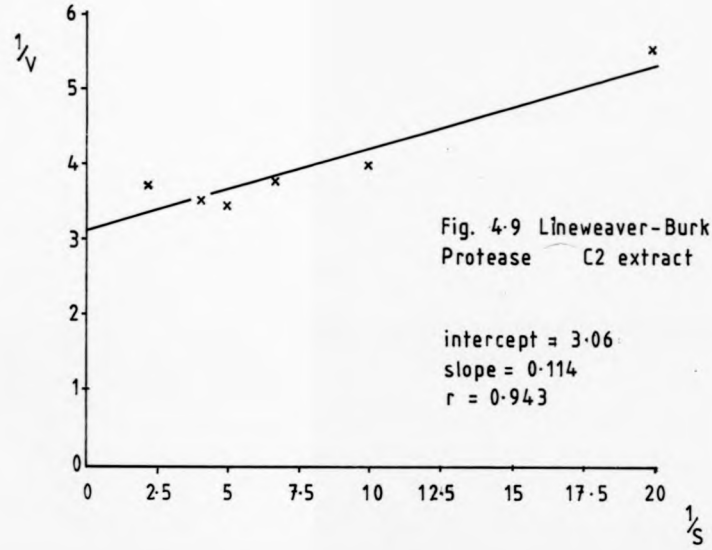


Fig. 4·9 Lineweaver-Burk plot
Protease C2 extract

intercept = 3·06
slope = 0·114
r = 0·943

The effect of toxic chemicals on saccharase from C4 and protease from C2 was checked by repeating assays under fixed conditions of substrate and sample size but with either varying concentrations of potassium cyanide ranging from 0 to 2 mg/l for saccharase and zinc sulphate from 0 to 100 mg/l zinc for protease. Each of the enzymes had a further check with single concentrations of a range of toxic chemicals.

Potassium cyanide at the concentrations used had no significant inhibitory effect. Zinc however inhibited protease causing an approximately logarithmic drop in activity with increasing zinc concentration.

Tables 4.19 and 4.20 list the results of experiments with different toxic chemicals.

4.6 PRELIMINARY IDENTIFICATION OF ISOLATED ORGANISMS

As detailed in Chapter 1 several authors have isolated and identified bacteria in aquatic habitats ranging from upland oligotrophic lakes and rivers to sewage sludge digestors, (Collins, 1963; Hendrie and Shewan, 1966; Whitthauer, 1980; and Nuttall, 1982). A varying number of taxonomic procedures were used with the object of constructing taxonomic schemes that would allow identification to species level.

The four bacteria isolated from the River Chelt and whose growth and production of enzyme activity have been described were subjected to an array of tests, as described in Chapter 2 the methods section, with an objective of placing them in genera.

Table 4.19

Effect of Toxic Chemicals on Saccharase in Extract of C4

Chemical	Concentration mg/l	Activity % of Control Without Inhibitor
Gas Oil	500	100%
$Zn\ SO_4$	100	9%
HCH	5	92%
Phenol	20	87%
Na Cl	5000	73%

Table 4.20

Effect of Toxic Chemicals on Protease in Extract C2

Chemical	Concentration mg/l	Activity % of Control Without Inhibitor
Gas Oil	1000	87%
KCN	5	75%
HCH	10	59%
Phenol	20	Interferes with assay
Na Cl	5000	72%

Results of the main tests are listed in Table 4.21 and show all 4 isolates to be gram negative, aerobic oxidative rods. In the dichotomous or branched key identification schemes developed by Hendrie et al (1966), Bergey 8th edition Buchanan and Gibbons (1974) and Witthauer, (1980) there is the possibility of more than one route to the genera Pseudomonas, Flavobacterium and to a lesser extent Aeromonas and Vibrio. This leads to some ambiguity between the Flavobacterium group and Pseudomonas for example if only a limited number of attributes are tested.

It has been pointed out that the genus Flavobacterium is really a heterogenous grouping of species producing yellow orange or red pigments most but not all of which are carotenoid in nature (Hendrie et al 1968; Weeks, 1969).

Three of the four isolates: C1, C2 and C4 are yellow/orange/pink pigmented and could therefore be placed in the Flavobacterium-Cytophaga-Flexibacter group which is a group containing a significant proportion of bacteria isolated from such aquatic environments. Growth and pigment production in response to culture on King's media fails to confirm the identify of any of the isolates as pigment producing Pseudomonads compared to the controls used: Pseudomonas aeruginosa and Pseudomonas fluorescens.

Colony appearance of C1 being somewhat spreading suggests a further characterisation to Flexibacter but the gliding motion of this genus was not observed. Cells in liquid medium cultures tended towards a slender and longer form again suggesting a Flexibacter species. The negative oxidase and apparent non

Table 4.21

Diagnostic Tests on Isolates C1, C2, C4 and D15

		Isolate			
		C1	C2	C4	D15
Colony appearance on CGY agar		Orange spreading	Pale orange discrete colonies	Pink small colonies	Non pigmented large colonies
Gram staining		- ve rods	- ve short rods	- ve short rods	- ve long rods
Motility		- ve	- ve	- ve	+ ve
Kovac's oxidase		- ve	- ve	- ve	+ ve
Hugh and Leifsons action on glucose		oxidative	oxidative	oxidative	oxidative
Carbohydrate utilisation LACTOSE		- ve	+ ve	- ve	+ ve
MALTOSE		+ ve	- ve	+ ve	+ ve
SUCROSE		+ ve?	+ ve	+ ve	+ ve
GLUCOSE		+ ve	+ ve	+ ve	+ ve
MANNITOL		- ve	- ve	- ve	+ ve
Citrate utilisation		- ve	- ve	- ve	+ ve
Catalase		+ ve	- ve	- ve	+ ve
Kanamycin resistance		reasonable growth	little growth	limited growth	no growth
Kings Medium	A	No growth	growth no colour	growth no colour	growth no colour
	B	growth no colour	growth no colour	growth no colour	growth colour poss

motility of the first three isolates tends not to support a grouping with Flavobacterium although the orange pink pigmentation is an important attribute supported by a resistance to the antibiotic kanamycin which would tend to exclude the possibility of an identification as Acinetobacter. The pattern of carbohydrate utilisation and the proteolytic action noted from previous experiments would support the Bergey classification of these isolates as Flavobacteria.

Organism D15 has several of the attributes of the genus Pseudomonas including motility, positive Kovacs oxidase, positive catalase and the obvious ability to utilise different carbon sources. Its lack of fermentative ability and proteolytic action would tend to exclude it from the genus Aeromonas.

Photographs of the isolates growing on CGY agar are presented in Plates 4.1 to 4.4.

Colony form and lack of floc type growth would rule out Zoogloea species and none of the isolates have the very short rods or cocci that would be associated with the Alcaligenes species.

To summarise isolates C1, C2 and C4 are placed in the Flavobacter group with the tentative further split of C1 as a Flexibacter and the other two as different Flavobacters. D15 has a sufficient number of characteristics of the Pseudomonas genus to be placed in that group.

Plate 4·1 Isolate C1 plated on CGY agar

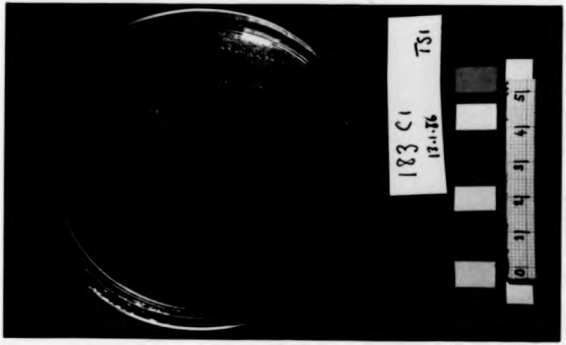


Plate 4·2 Isolate C2 plated on CGY agar

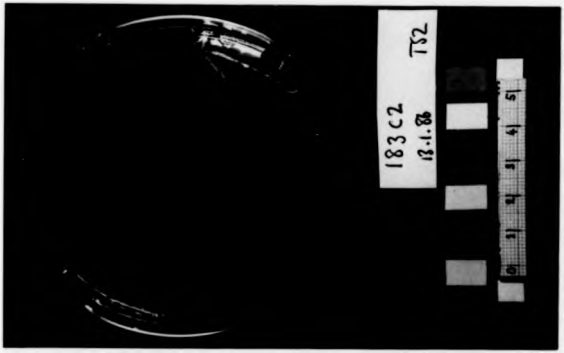


Plate 4·3 Isolate C4 plated on CGY agar

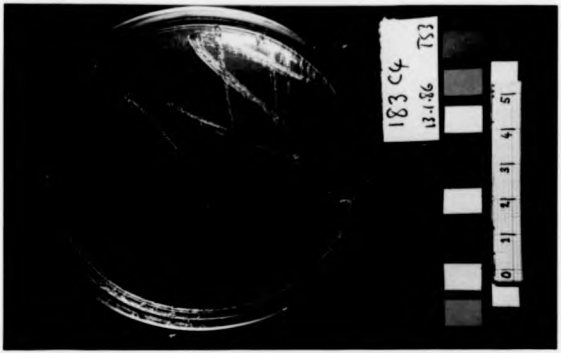
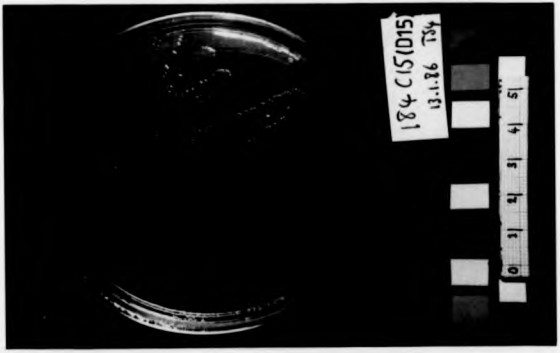


Plate 4·4 Isolate D15 plated on CGY agar



5 GENERAL DISCUSSION AND CONCLUSIONS

5.1 GENERAL DISCUSSION

5.1.1 Study Aims and Objectives

The overall aim of this study was to make measurements of hydrolytic enzyme activity in watercourses and sewage effluents in an area where no previous work on such activities was known to have been carried out and to investigate the role of enzyme activity in the process of self-purification.

Control of water pollution in the study area was the statutory responsibility of the Severn-Trent Water Authority under the provisions of inter alia the Water Act 1973, the Control of Pollution act 1974 and the Water Resources Act 1963. As a statutory body the Water Authority has a practical interest in any research which plans to have as an outcome an improved understanding of the process of self-purification of polluted waters.

Traditional work on self-purification of natural waters has concentrated on metabolism of organic matter in the water by heterotrophic organisms with consequent effects on dissolved oxygen, (Streeter and Phelps, 1925) macroinvertebrates (Kolkwitz and Marsson 1902) and fish life, (Hynes, 1960). More recently the study of oxygen and biodegradable organic matter balance in rivers has continued with ever more sophisticated models being produced (James, 1984). At the same time there has also been more analysis of the microbial ecology and mechanisms of heterotrophic activity in fresh and saline waters (Wright and Hobbie, 1965; Jones, 1985). Work on the cycling of matter in

the detritus system has hinted at the important role played by extracellular hydrolytic enzymes (Jorgensen, 1976) but work on such enzyme activity in natural waters, with the possible exception of the phosphomono and di esterases, is at an early stage.

On the other hand soil enzyme activity has been studied intensively for more than 30 years (Burns, 1978). It is probable that soil enzymes have been studied more intensively than aquatic enzymes because of their higher concentrations in soil samples. Most soil enzyme assays take about an hour to perform whereas comparable assays with water samples take at least 24 hours to perform with consequent problems of microbial growth and changes in physico-chemical factors during the incubation period. There has also been more emphasis placed by agriculturists on the fertility of soil and the relationship between hydrolytic enzyme activity, nutrient uptake and crop fertility. In spite of the large amounts of carbonaceous and nitrogenous wastes discharged from sewage treatment works to inland watercourses, estuaries and coastal waters, the relationship between hydrolytic activity, self-purification and fertility of waters has not been studied.

For soil enzymes the major areas of research have included the biochemistry of the interaction of enzymes in the turnover of organic matter, the use of enzyme activity as an index of soil fertility and the effect of agrochemicals on the biochemical activity of soils. All of these aspects have anologies in the watercourse, lake, oceanic and sediment environments.

Individual objectives of the study included the evaluation of enzyme assay methodologies in the context of the sites sampled and the operational concerns of the Water Authority.

Data from a preliminary survey of enzyme activities and bacterial counts was to be compared to the more traditional water quality parameters such as BOD and suspended solids.

An objective of practical value to the Water Authority would be the formulation of predictive models that were able to provide information on the pollution status of waters in terms of enzyme activity to compare with the traditional use of BOD.

A link between enzyme activity and heterotrophic bacteria has been reported by Griffiths (1983) and were this link to be confirmed, then the isolation of bacteria producing enzyme activity with a subsequent investigation of their nutritional requirements and biochemical properties of the enzymes produced might be expected to improve understanding of the role of such bacteria in the cycling of organic matter in the waters concerned.

5.1.2 Survey of Enzyme Activity in Natural Waters

5.1.2.1 Methodology

In the original selection of sampling sites a decision was made to take a watercourse system with a known polluting input of a scale that had a readily discernible effect on the receiving stream. A facility for measuring flows of both effluent and

river was also considered essential at an initial stage as flow is a fundamental feature of rivers. The River Chelt has a permanent gauging station operated by the Water Authority and the Hayden water reclamation works has its incoming flow continuously measured.

What was perhaps not fully appreciated at the outset of the survey was the extent of the variability of the values of enzyme activity and bacterial counts measured. BOD, suspended solids and flow also show inherent variability, particularly when rainfall is a determining factor as it is for flow and suspended solids, but these determinands are more gross indicators than single enzyme activities or plate counts of bacteria under restricted cultural conditions and could be expected to be less sensitive to small changes in microhabitats or input.

As an initial survey it may have been advantageous to limit sampling to a very small number of sites maybe only a single site which was chosen to be as limnologically simple as possible. Efforts would be made to locate and quantify all inputs that might have a significant and unpredictable effect on bacterial counts. These inputs would include such discharges as, treated sewage effluents, septic tanks, surface water sewers, storm sewage overflows, industrial discharges (including cooling waters), waste disposal sites and animal rearing units.

Sampling would be restricted to defined conditions of flow, probably low flow and attention paid to the seasonal effects. Low flow sampling should minimise the dynamic aspect of wet

weather discharges from urban areas, land run-off and the unpredictable resuspension of sediments within the system.

An alternative approach makes use of the relationship of required precision and sample numbers explained by Montgomery and Hart (1974) to overcome the variability by increasing sample numbers.

As a compromise in this study, data were pooled and then subsetted, to group together samples taken under similar conditions. In the subsequent analysis of the data only relationships found to be significant at the conventional confidence level of 5% or better were used to explain enzyme activity and scatterplots of data were always drawn to check for spurious conclusions.

Protease was selected for investigation because the breakdown of detrital proteinaceous matter is of basic importance in the nitrogen cycle. The casein substrate chosen is a natural protein although it would be unlikely to occur in natural waters apart from in unusual circumstances of polluting discharge or in waste water treatment works receiving effluent from dairy farms or milk processing industry. In situ proteolysis was not measured and this would be difficult to achieve without introducing substrate into the environment at such a concentration that substantial alterations were caused in the numbers and species make up of the microflora. Little et al (1979) measured the solubilisation and disappearance of the insoluble protein substrate hide-powder-azure retained in bags

suspended in lake water but noted that there was a significant increase in the numbers of proteolytic bacteria associated with the substrate after in situ incubation.

Sucrose as a substrate would be likely to be an uncommon component of the organic carbon content of natural waters. Being soluble and of relatively low molecular weight it might well be taken up directly by bacteria and other saprophytic micro-organisms. It may be on the other hand that absorption of sucrose is more efficient after its enzymic breakdown into monosaccharides in the region of the organism's cell envelope. If saccharase is predominately associated with cells it would follow that the saccharase activity detected in water samples is that either lost from cells or associated with cell components. Saccharase activity has been widely reported in soils (Burns, 1978) and has been detected in water samples taken in this study. Sucrose substrate concentration used in the assay was that reported by Verstraete et al (1976) but were high in relation to any concentration likely to be found in anything other than severe pollution conditions. This must be borne in mind when considering saccharase activity data.

Counting of bacteria was carried out in a simple manner. The assumption was made that the use of a nutrient medium developed by Pike et al (1972), which they found gave optimum recovery of heterotrophic bacteria from activated sludge, would give acceptable estimates of viable heterotrophic bacteria capable of growth on this same medium. No attempt was made to develop modifications of the medium or procedures to dismaggregate

particle-bound bacteria. This may explain some of the variability in viable counts. No total counts were carried out for comparison but the admittedly subjective differential counting based on colony morphology allowed some resolution of the variability in terms of enhanced correlation of certain colony types with enzyme activity or BOD.

Few of published reports of work on the relationship of bacterial flora, heterotrophic activity and limnological factors in natural waters, particularly rivers, include data on flows. Nuttall, (1982) included a flow term in multiple regression analysis when attempting to relate bacterial population estimates to 21 allegedly independent environmental variables in the Welsh River Dee. A study carried out by Baker and Farr (1977) concentrated on the relationship of counts of suspended bacteria, suspended solids and river discharge in two chalk streams concluding that there was a significant correlation between the factors measured over a two year period but that over a shorter timescale seasonal factors were introduced with added uncertainty caused by the high variability inherent in bacterial counts in rivers.

Other authors have stressed the importance of river discharge in determining the macroinvertebrate fauna in unpolluted waters (Jones and Peters, 1977); the inter-relation of discharge and stream morphology with a consequence in the wider context of conservation and stream use (Pirt, 1985; Beschta and Platts, 1986) and importantly, from the operational standpoint of the Water Authorities, the significance of flow and dilution in the

disposal of effluents to rivers (Garland, 1980).

To allow comparison of different sites where flow information was available the data were expressed as a proportion of an arbitrary low flow for the site. This allowed comparison but there was a risk of comparing "like with unlike", (for example a comparison of the large River Severn with the small River Chelt or the "artificial" Hayden sewage works). It is likely that more information on the relevance of flow to enzyme activity would have been obtained by concentration on single site data.

5.1.2.2 Data Interpretation

Empirical distributions of data for all determinands measured were seen to be non-symmetrical and in the cases of suspended solids and viable count they were highly skewed with extreme high values in a right hand tail. Taking logs of data allowed the fitting of a normal distribution and this distributional model is a reasonable one to use as an indicator of the types of statistical procedures to be adopted in line with the findings of Warn and Brew (1980). It may be that with reduction in the variability of raw data achieved by more stratified sampling this log normal model would be too coarse. An example would be where more complex distributions are seen (for example a multimodel effect of seasonal changes in activity). The nature of the underlying distribution assumes importance when summary statistics such as mean or fixed percentile have to be calculated and compared with standard values; for example in assessing compliance of effluent discharge quality with BOD standards.

The effect of variability in the data is seen strikingly when scatterplots are drawn. Seldom were clear cut relationships seen when enzyme activity and viable counts were plotted and outlying datapoints were common. It appeared that taking logs of the data went some way to suppressing the effect of extreme values.

Rank correlation coefficients quantified the relationships suggested by scatterplots but in the calculation of correlation coefficients extreme values can exert a disproportionate influence.

Both scatterplots and correlation coefficients showed a pattern of inter-relations between enzyme activity and the more conventional limnological factors measured. There were substantial differences between the clean Withy Bridge and dirty Boddington Bridge sites on the River Chelt. Mean saccharase activity was 7 times, protease activity 5 times, BOD 4 times and viable count 10 times higher at the dirty site. More reliable correlations were seen for the Boddington Bridge data which suggests that enzyme activity measurements may be a more accurate indicator of serious rather than mild organic pollution. Equally it may reflect lower variability in the data at those sites. This situation may be improved by increasing the sensitivity of the assays.

Some workers (Chrost et al 1986; Hoppe, 1983) have used chromogenic substrates, assaying coloured compounds by fluorimetry. Limits of detection are not reported but the use

of these substrates facilitates shortening of incubation times to obtain measurable breakdown of substrate and hence an assumed detection of lower intrinsic enzyme activities. This problem of low ambient activity was noted by Duddridge and Wainwright (1982) in clean river sediments. They increased activity by adding substrate to the sediment, incubating and then measuring activities. Amendment of clean river samples with either sucrose or casein resulted in measurable increases in both bacterial numbers and enzyme activity in proportion to the amount of substrate added. Amendment would disturb the habitat with changes to the microflora.

BOD measurements in oligotrophic waters tend to lack significance because of the inherent variability of the test and the disproportionate effects of blank readings when only small changes in dissolved oxygen are to be measured. The test is a crude measure of biodegradable matter and could not be expected to provide information on changes in microflora. A comparison of BOD and enzyme activities at the Withy Bridge and Boddington Bridge sites shows that for a three fold difference in BOD there is a six to seven fold difference in enzyme activity between the clean and dirty sites, revealing the potentially greater sensitivity of enzyme activity measurements.

If a sufficiently wide range of enzyme activities were to be measured, patterns of different activities may appear for environments containing different types and concentrations of pollutants (for example a protease to glycosidase ratio might represent the proportion of protein to carbohydrate substrate

present in a water or sediment and this ratio might further change as self-purification proceeds). Profiles of different enzyme activities may reflect the microflora at a site at that time with a higher protease activity indicative of the greater numbers of proteolytic bacteria.

The microflora in rivers will be partly a reflection of chemical and physical conditions upstream in the same way that macroinvertebrate populations have an integrating action in expressing the chemical quality of the catchment over a period of time. In contrast lake microflora more accurately reflect conditions where they actually grow.

Problems of inter-relatedness of the factors measured were much in evidence when a regression model was fitted to enzyme activity data. Saccharase and protease could be predicted by BOD, suspended solids, viable counts and to a lesser extent by flow and temperature. BOD was a predominant controlling factor and this relationship would be useful to the Water Authority if an enzyme assay were available that was of short duration and technically amenable to automation and could be used to give a measure of BOD of effluents and in receiving watercourses. A criticism of the assays used in this study is that with incubation times in excess of 24 hours the savings to the Water Authority of using these assays over conventional BOD tests would probably not justify the development work needed.

For an enzymatic method of pollution monitoring to be of practical value and if it were to stand any chance of being accepted as an alternative to the BOD test it would have to satisfy a wide range of criteria including: being universally applicable, reproducible, understandable by both expert and layman, being capable of execution within a few hours at most, amenable to the automated analytical techniques increasingly being introduced and being able to be used as a control parameter in waste treatment systems.

Regression equations presented in Chapter 3 had their predictive reliability confirmed but the small improvements achieved by use of combinations of factors could be seen. The question as to whether there is a single factor that would be a better predictor than combinations of BOD, suspended solids, viable count, nitrogen concentrations, flow or conductivity could not be resolved. other multivariate techniques such as principal component analysis mathematically group the effects of interrelated variables to minimise the number of explanatory variables.

The grouping of factors may simplify the use of predictive equations providing the factors grouped together for example BOD, viable count and ammonia can be linked in a limnological context.

Other manipulations of the preliminary survey data suggested that enzyme activity arose in part from the catchment. This observation arose from the fact of sampling under a wide range

of flow conditions when it would be expected that land run off, ingress of land subsurface-drainage and discharges of urban run-off would contain bacteria, decaying organic matter and enzymes, either free or particle-associated, washed directly out of soils.

Saccharase activity was found to be about 50% associated with particles retained on a GFC filter which is reported to have pore sizes of 1.2 microns on average, (Satoh, 1980). A comparable figure for protease was 35% and suggests that whilst many bacteria would pass a 1.2 micron filter, filtration through 0.45 or 0.2 micron filters would retain a significant majority of activity.

Following on from this is the nature of the relationship between enzyme activity in sediments and the water flowing over. No measurements were made on the enzyme activity of river sediments although this compartment of the river system is certain to be similar to lake sediments in being the site of much of the processing of organic matter derived either allochthonously or autochthonously, (Jones, 1985).

The modelling of the flux of organic matter and energy between sediment and water column would need to take full account of the hydraulics as well as the chemistry of the system.

5.1.3 Enzyme Activity of Organism Isolated from Environments Sampled

5.1.3.1 Methodology

Enzyme assays for these experiments were as for the preliminary survey modified only with respect to sample size, to avoid over or underestimation, and the omission of toluene in kinetic experiments where uncontrolled inhibitory effects must be excluded.

Moving the context of the study from natural environments to in vitro studies of isolated organisms involves a more analytical emphasis in the results and makes for more difficulty in interpreting observations.

Minimal development work on isolation or culture protocols beyond that needed to confirm that the basic plate count medium (Pike et al 1972) and a simple minimal medium (Flint and Hopton, 1976) were adequate to grow isolates, was carried out. No anaerobic organisms nor bacteria with exacting nutritional requirements were sought out.

Anaerobes would be of little importance in aerobic waters as they would be inactive. In sediments however they may be major producers of extracellular activity some of which could be transferred into the water column when flow increases cause resuspension of sediments. Crowther and Harkness (1975) reported the ten fold greater numbers of anaerobic as opposed to aerobic or facultative proteolytic bacteria in digesting sewage

sludge. They also reported that there was sufficient protease activity in digesting sludge to hydrolyse several times the daily input of protein. Enzyme activity may therefore be retained and transported into other compartments of the aquatic environment. It may be possible with the aid of suitable marker enzymes to trace protease activity through the human gut and into the natural environment. Other organisms could be sources of such activities.

A simplicity of approach was adopted with the objective of seeking gross effects that could be compared with the data from water samples.

5.1.3.2 Data Interpretation

An experiment detailed in Chapter 4.1 involving the addition of substrates to river water demonstrated a rapid increase in bacterial numbers and enzyme activities. Substrate addition stimulates production of enzyme and this would be expected in a natural system were there to be a sudden input of substrate. The presence of predators complicates the picture but produced the interesting observation that as the bacterial numbers fell there was a simultaneous reduction in enzyme activity suggesting a strong link between enzyme and cells. In natural systems and probably in an older culture the enzyme activity becomes dispersed between particulates and the water.

The random picking of colonies from agar plates with subsequent growth in a nutrient broth revealed that there was a wide range of growth and enzyme activities shown. Optical densities of some cultures suggests relatively low growth in the particular media used.

Using a minimal medium with different combinations of nitrogen sources, a glucose carbon source and supplementing with or substituting casein or sucrose as carbon or nitrogen sources, it was found that a proportion of the organisms were able to grow on the minimal medium in its simple form producing low specific saccharase and protease activities.

Addition of sucrose stimulated an increase in saccharase activity and often protease activity as well. No organism grew significantly with sucrose as sole carbon source which raises questions as to the role of saccharase enzymes produced by these bacteria when growing with glucose as carbon source. Casein however was able to act as sole carbon or sole nitrogen and in one case sole carbon and nitrogen source. The presence of casein stimulated the production of protease and in one case saccharase activity in addition. These experiments gave no clear indication of the mechanism of enzyme regulation apart from a confirmation that complex organic substrates stimulate the production of hydrolytic enzyme activity.

Organisms producing significant amounts of enzyme activity when grown in minimal medium supplemented with both sucrose and casein were grown up in that medium. Cells were harvested and used to produce crude extracts by disrupting cells. No attempt was made to fractionate or purify this crude extract which would be a mixture of intracellular enzyme and any activity associated with cell debris. Little activity was measured in the supernatant after centrifugation of cell suspensions.

Activity profiles for pH, temperature and the time of incubation along with kinetic experiments which gave a reasonable fit of Michaelis-Menten kinetics to the activity data complete the sequence of analysis linking measurements in natural environments to the more purely biochemical characterisation of extracted enzymes. Preliminary experiments on the effect of a range of toxic chemicals on the enzymes suggest that the two enzymes are affected to differing degrees by the same chemical.

More work on the effects on enzyme activities of toxic chemicals in both natural environments, by finding sites with biologically active concentrations of the chemicals, and in laboratory experiments on extracted and purified enzymes would add to the toxicological knowledge and could enable unsatisfactory discharges to be located or warning be given of potentially dangerous situations in rivers used for industrial or water supply abstraction.

5.2 CONCLUSIONS

The summarised conclusions are listed below and relate to the aims of the study listed on page 93.

1 Published methods for measuring reducing sugars and tyrosine were tested with the buffer system used in the study. The release of reducing sugar from sucrose and tyrosine from casein incubated with samples of natural waters was measured at a range of temperatures, pH and times of incubation. (Chap 2.2.1, 2.2.2). Assay conditions under which hydrolysis of added substrate was maximum were determined. Replicates of assays had acceptable reproducibilities (p 112, 105).

2 Samples of water from the River Chelt were collected at five sites and also from the Hayden water reclamation works fully treated discharge to the river over a period of 50 months. One main river site was 800 metres upstream and the other 750 metres downstream of the treated sewage discharge. Other samples were taken of watercourses and waste waters with differing degrees of organic pollution. A more detailed description of the sites was presented in Tables 2.1 and 3.1. All samples assayed for protease showed measurable activity with higher activity in the more polluted water. For saccharase 5 out of 22 samples at the main unpolluted site had activity at or below the limit of detection of the technique. Both enzymes had higher activity at the more

organically polluted sites expressed as BOD.

3 Data on enzyme activities, chemical determinands, discharge and bacterial counts expressed as colony forming units were found to show wide variability. On constructing histograms and probability plots it was found that a log-Normal was a suitable empirical model of data distribution (p 148 Chap 3.1.2).

4 A non-parametric method of rank correlation was used to identify relationships between enzyme activity and other limnological or bacterial factors. At specific sites there was a positive correlation between both enzymes and BOD. For sub-sets of pooled data, representing activity under restricted environmental conditions such as high or low temperature, good positive correlations with BOD bacterial numbers expressed as colony forming units and particulate matter. Under low temperature conditions there was in addition a negative correlation with discharge (Table 3.8 to 3.14).

5 Multiple regression equations were formulated to express enzyme activity in terms of other limnological and bacterial factors. At the Withy Bridge site the equation relating saccharase to BOD had a coefficient of determination of 77%; for the high temperature data set the equation relating saccharase to suspended solids had a coefficient of determination of 71%. Inclusion of other predictor

variables or the log transformation of variables only marginally increased the fit of the variables. (Table 3.32). Protease generally was modelled less reliably although log transformation improved the coefficient of determination as did the inclusion of other predictors. The ability of regression equations to predict enzyme activity at different sites was tested. Expressions with multiple factors were found to give satisfactory prediction and these expressions were not necessarily the ones with the best fit at the original sites (Fig 3.37 - 3.38).

6 Heterotrophic bacteria present in water samples and selected as examples of producers of enzyme activity were isolated from complex solid media used for plate counts. Identification to genus level was carried out on 4 isolates using standard procedures (Chap 2.3.1.3) with tentative assignment to the genera: Pseudomonas. Flavobacterium (2 isolates) and Flexibacter. These genera are commonly found in natural waters. (Chap 1.1.2).

7 Bacteria isolated were grown first in liquid complex medium with or without the addition of casein or sucrose (Tables 4.6, 4.7). Organisms producing enzyme activity under these cultural conditions were sub-cultured in a mineral salts medium with a glucose carbon source substituting casein or sucrose as carbon sources. Approximately 20% of the original isolates grew under these conditions. Addition of sucrose stimulated production of saccharase and in some

cases protease activity. In no case could sucrose act as a sole carbon source at the concentration and cultural conditions employed. This observation contrasts with the result of experiment is Chap 4.1 where addition of sucrose to river water caused an increase in bacterial numbers expressed as colony forming units and saccharase activity. (Fig 4.1 - 4.2).

Casein addition stimulated protease production and in two cases casein could act as sole carbon and nitrogen source. These preliminary experiments demonstrated that the presence in the medium of enzyme substrates could stimulate production of hydrolytic enzyme activity but no clear indication as to the mechanism of enzyme regulation was provided (Chap 4.3.2.2).

8 Cultures of organisms producing enzyme activity were grown and crude extracts were made by disrupting the cell suspensions by ultrasonication. Enzyme activity was predominantly associated with the cell suspension and cell extracts (Chap 4.5.1).

9 The crude extracts were not purified or fractionated but were found to have pH and temperature profiles of activity for both saccharase and protease (Table 4.17). There was a linear increase in hydrolysis of added casein or sucrose substrate with prolonged incubation.

The effect of a range of substrate concentrations on the rate of enzyme hydrolysis was found to be modelled with acceptable confidence by Michaelis-Menten kinetics. (Fig 4.8, 4.9). In preliminary experiments saccharase and protease were seen to have differing sensitivities to a range of toxic chemicals.

Ackers, P, 1985 in Advances in Water Engineering, Ed. Tebbutt, T.H.Y., Elsevier Applied Science, London. Proceedings of the International Symposium "Advances in Water Engineering".

Adamse, A.D., 1968, Water Res., 2, 665-671. Formation and final composition of the bacterial flora of a dairy waste activated sludge.

Albright, L.J. and Wentworth, JW., 1973, Enviro, Pollut. 5, 59-72. Use of the heterotrophic activity technique as a measure of eutrophication.

Allen, L.A., 1944, J Hyg. Camb. 43, 424-431. The bacteriology of activated sludge.

Anon 1934, Bacteriological Examination of Water Supplies, Report No 71, Ministry of Health. H.M.S.O. London.

Anon., 1961. Report of the Commission on Enzymes of the International Union of Biochemistry. Pergamon Press, London.

Anon., 1972. Analysis of Raw Potable and Waste Waters. Department of the Environment. HMSO London

Anon, 1978, National Council of the Paper Industry for Air and Stream Improvement Tech. Bull., 308. New York. A study of the fate of biosolids from biologically treated effluent in laboratory constructed streams.

Anon, 1983. Reports on Public Health and Medical Subjects No 71. Methods for Examination of Waters and Associated Materials. The Bacteriological Examination of Drinking Water Supplies, 1982. H.M.S.O., London.

Ardern, E. and Lockett, W.T. 1914, 33. 525-530. Experiments on the oxidation of sewage without the aid of filters.

Ashley, N.V. and Hurst, T.J., 1981, Wat. Res., 15. 633-638. Acid and alkaline phosphatase activity in anaerobic digested sludge: a biochemical predictor of digester failure.

Ashley, R.P. and Lloyd, J.W. 1978 Journal of Hydrology 39, 355-364. An example of the use of factor analysis and cluster analysis in groundwater chemistry interpretation.

Baker, J.H. and Farr, I.S. 1977, Arch. Hydrobiol. 80, 308-326. Orgins, characterisation and dynamics of suspended bacteria in two chalk streams.

Baker, J.H. and Morita, R.Y., 1983, Environmental Pollution (Series A), 31, 149-157. A note on the effects of crude oil on microbial activities in a stream sediment.

Bailey, N.T.J., 1959. Statistical Methods in Biology. The English Universities Press, London.

Bagenal, M., 1955, J. Mar, biol. Assoc. U.K. 34, 289-296. A note on the relations of certain parameters following a logarithmic transformation.

Barnes, R.K. and Mann, K.H. 1980 Eds. Fundamentals of Aquatic Ecosystems. Blackwell Scientific Publications, Oxford.

Batistic, L., Sarkar, J.M. and Mayaudon. J., 1980, Soil Biol. Biochem. 12, 59-63. Extraction, purification and properties of soil hydrolases.

Bell, C.R. and Albright, L.J. 1981, Mar. ecol. Prog. Ser. 6, 317-325. Attached and Free-Floating bacteria in the Fraser River estuary. Biol. abstr, 73, 59826, (1982).

Bell, C.R., Holder-Franklin, M.A. and Franklin, M. 1980. Wat. Res. 14 449-460. Heterotrophic bacteria in two Canadian rivers - 1. Seasonal variations in the predominant bacterial populations.

Berman, T., 1970, Limnol. Oceanogr., 15. 663-674. Alkaline phosphatases and phosphorus availability in Lake Kinneret

Beschta, R.L. and Platts, W.S. 1986. Water Resources Bulletin, 22, 369-379. Morphological features of small streams: significance and function.

Billen, G. 1982. Heterotrophic utilisation and regeneration of nitrogen. In Hobbie, J.E. and Williams, P.J. Le B. Eds. Heterotrophic Activity in the Sea. Plenum Press.

Bitton, G., 1983, CRC Critical reviews in environmental control, 13, 51-67. Bacterial and biochemical tests for assessing chemical toxicity in the aquatic environment: a review.

Boavida, M.J. and Heath, R.T., 1984, Limnol. Oceanogr., 29. 641-645. Are the phosphatases released by Daphnia magna components of its food.

Bonde, G.J. 1966. Proceedings of the third International Conference on Water Pollution Research (Munich) 3 87-103. Heterotrophic bacteria in a polluted marine environment.

Breure, A.M., Mooijman, K.A. and van Andel, J.G., 1986, Appl. Microbiol. Biotechnol., 24, 426-431. Protein degradation in anaerobic digestion: influence of volatile fatty acids and carbohydrates on hydrolysis and acidogenic fermentation of gelatin.

Brown, C.M., and Stanley, S.O, 1972, J. APPL. CHEM. BIOTECHNOL, 22 363-389. Environmental mediated changes in the cellular content of the "pool" constituents and their associated changes in cell physiology.

Brown, G.W., 1976, J. Fish. Res. Board Can., 33, 2018-2022. Effects of polluting substances on enzymes of aquatic organisms.

Buchanan, R.E. and Gibbons, N.E. Ed., 1974, Bergey's Manual of Determinative Bacteriology 8th Edition.

Buck, T.C., Keefer, C.E, and Hatch, H., 1953, Sew, and Ind, Wastes, 25, 993-1002. A facultative anaerobe isolated from digesting sludge.

Burns, R.G. Ed. 1978. Soil Enzymes. Academic Press, London

Butterfield, C.T. and Wattie, E., 1941, U.S. Public Health Reports. 56 2445-2464 Washington. Studies of sewage purification XV: Effective bacteria in purification by trickling filters.

Butterfield, C.T., 1935, U.S. Public Health Reports, Washington, 50, 1935, 671-684. Studies of sewage purification II. A zoogloea forming bacteria isolated from activiated sludge.

Cairns, J. (ed), 1971. The Structure and function of freshwater microbial communities. Researc Division Monograph. 3, Virginia Polytechnic Institute and State University, Blacksburg.

Carney, J.F. and Colwell, R.R., 1976, Appli. envir. Microbiol., 31, 227-231. Heterotrophic utilisation of glucose and glutamate in an estuary: effect of season and nutrient load.

Carroad, P.A. and Wilke, C.R., 1978. Resource Recovery and Conservation, 3, 165-178. Enzymes and micro-organisms in food industry waste processing and conversion to useful products: a review of the literature.

Chan, K. and Leung, S.L., 1986, Bull. Environ. Contam. Toxicol., 36, 52-59. Effects of Paraquat and Glyphosate on growth, respiration and enzyme activity of aquatic bacteria.

Christenson, G.M., Olson, D. and Riedel, B., 1982, Environmental research, 29, 247-255. Chemical effects on the activity of eight enzymes: A review and a discussion relevant to environmental monitoring.

Chrost, R.J. and Krambeck, H.J. 1986. Arch. Hydrobiol. 106, 79-90. Fluorescence correction for measurement of enzyme activity in natural waters using methylumbelliferone substrates.

Chrost, R.J., Wecslo, R. and Halemejko, G.Z., 1986, Arch. Hydrobiol., 107, 145-165. Enzymatic decomposition of organic matter by bacteria in an eutrophic lake.

Collins, V.G., 1963, Proceedings of Society for Water Treatment and Examination 12 40-66. The distribution and ecology of bacteria in freshwater.

Cookson, J.T. and Burbank, N C., 1965, J. Wat. Poll. Control Fed., 37, 822-841. Isolation and identification of anaerobic and facultative bacteria present in the digestion process.

Cooper, R.A. and Weekes, A.J. 1983, Data Models and Statistical Analysis, Philip Alan, Oxford.

Corpe, W.A. and Winters, H. 1972, Can. J. Microbiol. 18, 1483-1490. Hydrolytic enzymes of some periphytic marine bacteria.

Crabtree, K and McCoy, E., 1967. Int. J. Syst. Bacteriol. 17, 1-10. Zoogloea ramigera Itzigsohn, identification and description.

Crawford, C.C., Hobbie, J.E. and Webb, K.L., 1974, Ecology. 55, 551-563. The utilisation of dissolved free amino acids by estuarine micro-organisms.

Crowther, R.F. and Harkness, N in: Ecological Aspects of Used Water Treatment. Ed. Curds, C.R. and Hawkes, H.A. 1975. Vol. 1. The Organisms and their Ecology. Academic Press, London.

Cruickshank, R., Duguid, J.P. and Swain, R.H.A. 1968, 650-651. Medical Microbiology

Currie, D.J., Bentzen, E. and Kalff, J., 1986, Can. J. Fish. Aquat. Sci., 43, 311-318. Does algal-bacterial phosphorus partitioning vary among lakes? A comparative study of orthophosphate uptake and alkaline phosphatase activity in freshwater.

Curtis, E.J.C., 1969, Water Res., 3, 289-311. Sewage fungus: its nature and effects.

Daatselaar, M.C.C. and Harder, W., 1974. Arch. Microbiol., 101, 31-34. Some aspects of the regulation of the production of extracellular proteolytic enzymes of a marine bacterium.

Daubner, I, et al 1981, Zentralbl. Bakteriol. Mikrobio. Hyg., Abt. 174, 453-457. Ability of intestinal bacteria to multiply in water. Biol Abstr 74 86494 1982.

Dias, F.F. and Bhat, J.V., 1964, Appl. Microbiol. 12, 412-417. Microbial ecology of activated sludge I. Dominant bacteria.

Dixon, M. and Webb, E.C. 1967. Enzymes, 2nd Edition. Longmans Green, London

Domokos, L, et al 1981, Rev. Ig. Bacterial Virusol, Parazitol. Pneumoftiziol. Ser. Ig., 30 137-144. Aeromonas hydrophila strains isolated from river water. Biol Abstr. 74 35422 1982.

Drake, C.H. 1966 Health Laboratory Service. 3, 10. Evaluation of culture media for the isolation and enumeration of Pseudomonas aeruginosa.

Duddridge, J.E. and Wainwright, M. 1980, Environmental Technology Letters. 1, 319-326. Effect of sodium chloride on enzyme activity and synthesis in river sediments.

Duddridge, J.E. and Wainwright, M., 1981, Environmental Technology Letters, 2, 75-80. Enzyme activity measurements as a means of assessing a river sediment's capacity to degrade organic pollutants.

Duddridge, J.E. and Wainwright, M. 1982. Water Res. 16, 329-334. Enzyme activity and kinetics in substrate amended river sediments.

Dudzik, M., Harte, J., Jassly, A., Lapan, E., Levy, D and Rees, J., 1979, Intern. J. Environmental Studies, 13, 125-130. Some considerations in the design of aquatic microcosms for plankton studies.

Duguid, J.P. and Wilkinson, J.F. 1953, J. gen. Microbiol, 19 174-189. The influence of cultural conditions on polysaccharide production by Aerobacter aerogenes.

Dunnill, P. and Rudd. M. 1984 Biotechnology and British Industry. Science and Engineering Research Council, Swindon.

Einstein, H.A., 1982, in River Ecology and Man Eds. Oglesby, R.T., Carlson, C.A. and McCann, J.A. Academic Press, London.

Fenchel, T.M. and Jorgensen, B.B., 1977 in Advances in Microbial Ecology, Ed. Alexander, M. Plenum Press N. York. Detritus food chains of aquatic ecosystems: The role of bacteria.

Flint, K.P. and Hopton, J.W., 1977, Eur. J. Appl. Microbiol. 4, 205-215. Seasonal variation in the phosphatase activity of waters and sewage sludges.

Flint, K.P. and Hopton, J.W., 1976, European. J. Appl. Microbiol. 3, 237-243. Some properties of a neutral phosphatase of a bacterium isolated from a trickling filter effluent.

Flint, K.P. and Hopton, J.W., 1977, Eur. J. Appli. Microbiol. 4, 195-204. Substrate specificity and ion inhibition of bacterial and particle association alkaline phosphatases of waters and sewage sludges.

Florkin, M. and Stotz, E.H. (eds) 1964. Vol. 13. Comprehensive Biochemistry. Elsevier, New York.

Frankland, G.C., and Frankland P.F., 1894, Micro-organisms in water, their significance, identification and removal, Longmans, Green and Co., London.

Fred, E.B., Wilson, F.C., and Davenport, A. 1924, Ecology, 5 322-331. The distribution and significance of bacteria in Lake Mendota.

Friant, S.L., Patrick, R and Lyons, L.A. 1980, Journal Wat. pollut. Control. Fed., 52, 351-363. Effects of non settleable biosolids on stream organisms.

Frost, W.H., and Streeter, H.W., 1924, Report of Survey and Laboratory Studies, Public Health Bulletin 143. A Study of the Pollution and Natural Purification of the Ohio River, Part II.

Garland, J.H.N. 1980, Effluent and Water Treatment Journal, 20, 71-74. The importance of flow measurement.

Gaudy, A.F., Jr., and Gaudy, E.T., 1966, Ann. Rev. Microbiol. 20, 319-332. Microbiology of waste waters.

Golterman, H.L., 1963, Mitt. Internat. Verein. Limnol. 14, 25-30. Investigations on photosynthesis and mineralisation in experimental ponds.

Golterman, H.L., 1975. Physiological Limnology. Elsevier Scientific Publishing, New York.

Goulder, R., Blanchard, A.S., Sanderson, P.L. and Wright B 1979, J. appl. Bact. 46 285-289. A note on the recognition of pollution stress in populations of estuarine bacteria.

Goulder, R., Blanchard, A.S., Sanderson, P.L. and Wright. B., 1980. Water Res., 14, 591-601. Relationships between heterotrophic bacteria and pollution in an industrialised estuary.

Griffiths, R.P., Caldwell, B.A., Broich, W.A. and Morita, R.Y., Appl. Environ. Microbiol., 1982 42, 792-801. Long-term effects of crude oil on uptake and respiration of glucose and glutamate in Arctic and Subarctic marine sediments.

Griffiths, R.P., Caldwell, B.A., Broich, W.A. and Morita, R.Y., 1982, Marine Pollution Bull., 13, 273-278. Long term effects of crude oil on microbial processes in subarctic marine sediments. Studies on sediments amended with organic nutrients.

Griffiths, R.P., 1983, Marine Pollution Bulletin, 14, 162-165. The importance of measuring microbial enzymatic functions while assessing and predicting long term anthropogenic pertubations.

Griffiths, R.P., Caldwell, B.A., Broich, W.A. and Morita, R.Y., 1983, Mar. Ecol. Prog. Ser. 10, 265-275. Microbial processes relating to carbon cycling in South eastern Bering Sea sediments.

Halemejko, G.Z. and Chrost, R.J. 1986. Arch. Hydrobiol. 107, 1-21. Enzymatic hydrolysis of proteinaceous particulate and dissolved material in an eutrophic lake.

Hall, K.S., Kleiber, P.M. and Yesaki, I., 1972, Mem. Inst. Ital. Idrobiol., Suppl. 29, 441-471. Heterotrophic uptake of organic solutes by micro-organisms in the sediment.

Hamence, J.H., 1967, J. Association Public Analysts, 5, 88-100. The composition of muds from rivers, estuaries and lakes.

Hankin, L. and Sands, D.C., 1974. J. Wat. Pollut. Cont. Fed. 46, 2015-2025. Bacterial production of enzymes in activated slusge systems.

Hankin, L., Stephens, G.R. and Hill, D.E., 1979. Canadian J. Microbiol., 25, 1258-1263. Effect of additions of liquid poultry manure on excretion of degradative enzymes by bacteria in forest soil and litter.

Harrison, M.J., Wright, R.T and Morita, R.Y., 1971, Appl. Microbiol., 21, 698-702. Method for measuring mineralisation in lake sediments.

Hasler, A.D., 1947, Ecology, 28, 383. Eutrophication of lakes by domestic drainage.

Hattingh, W.H.J., 1963, Wat. Waste Treat. J., 9 476-480. Activiated sludge studies - 3. Influence of nutrition on bulking

Heath, R.T. and Cooke. G.D., 1975. Verh. Internat. Verein. Limnol., 19, 959-965. The significance of alkaline phosphatase in a eutrophic lake.

Heath, R.T., 1979, Intern. J. Environmental Studies. 13, 87. Holistic Study of an Aquatic Microcosm: Theoretical and Practical Implications.

Hendrie, M.S. and Shewan, J.M. 1966. The identification of certain Pseudomonas species. Identification Methods for Microbiologists Part A, Society for Applied Bacteriology Technical Series 1. ed. Gibbs, B.M. and Skinner, F.A. 1-7 Academic Press, London.

Hendrie, M.S., Mitchell, T.G. and Shewan, J.M. 1968. The identification of yellow pigmented rods in: Identification Methods for Microbiologists Part B, Society for Applied Bacteriology Technical Series 2 ed Gibbs, B.M. and Shapton, D.A. Academic Press, London.

Hendrie, M.S., Holding, A.J. and Shewan, J.M. 1974. International Journal of Systematic Bacteriology. 24 534-550. Amended description of the genus Alcaligenes and Alcaligenes faecalis and a proposal that the generic name Achromobacter be rejected: status of the named species of Alcaligenes and Achromobacter.

Hendricks, C.W., 1974, Applied Microbiol, 28, 572-578. Sorption of heterotrophic and enteric bacteria to glass surfaces in the continuous culture of river water.

Hendry, G.S. et al 1982, Water Res. 16 1291-1299. Some effects of pulp and paper wastewater on microbiological quality of a river.

Henrici, A.T., 1938, J. Bact., 35 129. Studies of freshwater bacteria. IV - Seasonal fluctuations of lake bacteia in relation to plankton production.

Herbes, S.E., 1981, Appl. Environ. Microbiol. 41, 20-28. Rates of microbial transformation of polycyclic aromatic hydrocarbons in water and sediments in the vicinity of a coal-coking wastewater discharge.

Hill, A.R., 1982, Freshwater Biol., 12, 293-298. Phosphorus and major cation mass balances for two rivers during low summer flows.

Hobbie, J.E. and Crawford, C.C., 1969, Limnol. Oceanogr. 14, 528-532. Respiration corrections for bacterial uptake of dissolved organic components in natural waters.

Hobbie, J.E., Daley, R.J., and Jasper, S. 1977, Appl. Environ. Microbiol. 33 1225-1228. Use of Nucleopore filters for counting bacteria by fluorescence microscopy.

Hobson, P.N. and Shaw, B.G., 1971 in Microbial Aspects of Pollution. Soc. Appl. Bact. Symp. Ser. No.1 - Eds. Sykes, G. and Skinner, F.A. Academic Press.

Holm-Hansen, O., and Booth, C.R., 1966, Limnol. Oceanogr. 11 510-519. The measurement of adenosine triphosphate in the ocean and its ecological significance.

Holm-Hansen, O., Sutcliffe, W.H. and Sharpe, J. 1968, Limnol. Oceanogr. 13 507-514. Measurement of deoxyribonucleii acid in the ocean and its ecological significance.

Holeman, J.N., 1968, Water Resources Research, 4, 737-747. The sediment yield of major rivers of the world.

Hoppe, H-G. 1983. Mar. Ecol. Prog. Ser. 11, 299-308. Significance of exoenzymatic activities in the ecology of brackish water: measurements by means of methylumbelliferyl-substrates.

Hopton, J.W., Wat. Res. 1970, 4 493-499. A survey of some physiological characteristics of stream-borne bacteria.

Hugh, R., and Leifson, E., 1953, J. Bact 66 24-25. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria.

Hutchinson, G.F., 1975. A Treatise on Limnology, III Limnological Botany, Wiley, New York.

Hynes, H.B.N., 1960. The Biology of Polluted Waters. Liverpool University Press.

Iturriaga, R., 1979, Marine Biology, 55, 157-169. Bacterial activity related to sedimenting particulate matter.

Itzigsohn, H. 1868, S.B. Gesellschaft naturf Freunde, Berlin. Entwicklungsvorgange von Zoogloea, Oscillaria, Synedra, Staurastrum, Spirotaenia and Chroolepus.

Jackman, D.M., Bartlett, F.M. and Patel, T.R. 1983. Applied and Environmental Microbilogy. 46, 6-12. Heat stable proteases from psychtrophic Pseudomonads: comparison of immunological properties.

Jacob, F. and Monod, J., 1961, J. Mol. Biol., 3, 318-356. Genetic regulatory mechanisms in the synthesis of proteins.

James, A. 1964, J. Appl. Bact. 27, 197-207. The bacteriology of trickling filters.

James, A. 1984 Modelling of fixed film reactors in James, A. Ed. An Introduction to Water Quality Modelling. John Wiley and Sons.

Janicki, A and De Costa, J. 1979 Arch. Hydrobiol. 85, 465-481. A multivariate analysis of the crustacean plankton community of an acid reservoir.

Jannasch, H.W., 1969, Verhandlungen der Internationalen Vereingung der theoretische and angewandte Limnologie, 17 25-39. Current concepts in aquatic microbiology.

Jannasch, H.W., 1958, J. Gen. Microbiol. 18, 609-620. Studies on planktonic bacteria by means of a direct membrane filter method.

Joint, I.R. and Pomroy, A.J., 1982, J. exp. mar. Biol. Ecol, 58, 33-46. Aspects of microbial heterotrophic production in a highly turbid estuary.

Jones, H.R. and Peters, J.C. 1977 Water Research Centre, Technical Report TR 41. Physical and biological typing of unpolluted rivers. Water Research Centre.

Jones, J.G., 1970. J. Appl. Bact., 33 679-686. Studies on freshwater bacteria. Effect of medium composition and method on estimates of bacterial populations.

Jones, J.G., 1972, J. Ecol., 60, 777-791. Studies of freshwater micro-organisms: phosphatase activity in lakes of differing degress of eutrophication.

Jones, J.G. 1973. J. appl. Bact. 36, 197-210. Use of Nonparametric Tests for the anlysis of data obtained from preliminary surveys: a Review.

Jones, J.G., 1979 a, J. Gen. Microbiol. 115, 19-26. Microbial activity in lake sediments with particular reference to electrode potential gradients.

Jones, J.G., 1979 b, Freshwater Biological Association Scientific Publication No. 39. A guide to methods for estimating microbial numbers and biomass in fresh water.

Jones, J.G., 1980, J. Gen. Microbiol. 177 285-292. Some differences in the microbiology of profundal and littoral lake sediments.

Jones, J.G., 1985, Decomposition in lake sediments: bacterial action and interaction in Fifty-third Annual Report 1985. Freshwater Biological Association, Ambleside.

Jones, J.G., and Simon, B.M., 1975, J. Appl. Bact., 39, 317-329. An investigation of errors in direct counts of aquatic bacteria by epifluorescence microscopy, with reference to a new method for dyeing membrane filters.

Jones, K.L. and Grainger, J.M. 1983, Eur. J. Appl. Microbiol. Biotechnol. 18, 181-185. The application of enzyme activity measurements to a study of factors affecting protein, starch and cellulose fermentation in domestic refuse.

Jorgensen, C.B., 1976, Biol. Rev. 51, 291-328. Mordern ideas on the use of dissolved organic matter in aquatic environments.

Joung, H.M., Miller, W.W., Mahannah, C.N. and Guitjens, J.C. 1979 J. Environ. Qual. 8, 95-100. A generalised water quality index based on Multivariate factor analysis.

Kabler, P., 1959, Sew. and Ind. wastes., 31, 1373-1382. Removal of pathogenic micro-organisms by sewage treatment processes.

Kakde, S.N. and Raman, V. 1978 IAWPC Tech. Annual-V 139-152. Enzyme as an index of activity of waste waters and their biomass.

Keeble, J.R., and Cross. T. 1977, J. Appl. Bacteriology, 43, 325-327. An improved medium for the enumeration of Chromobacterium in soil and water.

Keiko, K-H., 1986, Wat. Res., 20, 491-494. Inhibition of the activities of B-galactosidase and dehydrogenases of activated sludge by heavy metals.

Kelly, M. and Clarke, P.H. 1962, J. Gen. Micribiol. 27, 305-316.

Kim, J. and Zobell, C.E. 1974 pp 368-385. Occurrence and activities of cell free enzymes in oceanic environments in Colwell, R.R. and Morita, R.Y. (eds). Effect of the Ocean Environment on Microbial Processes. University Park Press, New York 1974.

King, E.O., Ward, M.K., and Raney, D.E., 1954. Journal of Laboratory and Clinical Medicine. 44, 301-307. Two simple media for the demonstration of pyocyanin and fluorescein.

Kirchman, D. and Mitchell, R., 1982, Appl. Environ, Microbiol., 43, 200-206. Contribution of particle bound bacteria to total micro-heterotrophic activity in five ponds and two marshes.

Kiss, S., Dragan-Bularda, M. and Radulescu. D. 1978. Soil polysaccharadases: activity and agricultural importance. In Burns, R.G. Ed. Soil Enzymes. Academic Press, London.

Kobayashi, K., Hashimoto, S., Fujiwara, K., Haraguchi, H., Fuwa, F. and Otsuki, A., 1984, Chemistry Letters. 565-568. Correlations between dissolved alkaline phosphatase and orthophosphate in lake water.

Koch, R. 1884, Berlin Klin Wochenschr, 21, 477-483. Conferz zur Erorterung der Cholerafrage

Kolkwitz, R. and Marsson, M., 1902. Mitt. a.d. Kg. Prufungsanst, f, Wasserversorg.u., Abwasserbeseitigung zu Berlin 1, 33-72. Grundsatze fur die biologische Beurtielung des Wassersnach seiner Flora und Fauna.

Kovacs, N., 1956, Nature, London, 178, 703-703. Identification of Pseudomonas pyocyanea by the oxidase reaction.

Kuznetsov, S.I., Dubinia, G.A. and Lapteva, N.A., 1979, Ann. Rev. Microbiol., 33, 377-387. Biology of oligotrophic bacteria.

Ladd, J.N. and Butler, J.H.A., 1972, Soil Biol. Biochem. 4, 19-30. Short-term assays of soil proteolytic enzyme activities using proteins and dipeptide derivatives as substrates.

Leopold, L.B., Wolman, M.G. and Miller, J.P., 1964. Fluvial Processes in Geomorphology. W.H. Freeman. San Francisco.

Lenhard, G., 1967, Hydrobiologia, 29, 67-79. Determination of protease activity in bottom deposits of sewage stabilisation ponds.

Lenhard, G., 1969. Hydrobiologiya, 33, 193-200. Determination of urease activity in biological purification systems.

Lighthart, B. and Oglesby, R.T., 1969, J. Wat. Pollut. Control. Fed. 41, 267-281. Bacteriology of an activated sludge wastewater treatment plant - a guide to methodlogy.

Lin, S., Evans, R.L. and Beuscher, D.B., 1974, Applied Microbiology 28, 288-297. Bacteriological Assessement of Spoon River Water Quality.

Little, T.E., Sjogren, R.E. and Carson, G.R. 1979. Applied and Environmental Microbilogy. 37, 900-908. Measurement of proteolysis in natural waters.

Lowry, O.H., Rosenbourgh, N.J. and Randall, R.J. 1951. J. Biol. Chem. 193, 265-275. Protein measurement with Folin-phenol reagent.

Lynch, J.M., and Poole, N.J. Ed. 1979, Microbial Ecology: A Conceptual Approach, Blackwell Scientific Publications.

Manczak, H. and Florczyk. H. 1971. Wat. Res. 5, 575-584. Interpretation of results from studies of pollution of surface flowing waters.

Mann, K.H. in River Ecology and Man., 1972. Eds. Oglesby R.T., Carlson, C.A. and McCann, J.A. Academic Press.

Marshall, K.C., 1976, Interfaces in Microbial Ecology. Harvard University Press. Cambridge. U.S.

Marshall, K.C., 1978, in Water Pollution Microbiology Vol 2. Ed. Mitchell, R., Wiley Interscience. The effects of surfaces on microbial activity.

Matavalj, M., and Flint, K.P. 1987 Microbial Ecology 13, 141-158. A model for acid and alkaline phosphatase activity in a small pond.

McKinney, R.E. and Weichlein, R.G. 1953, Appl. Microbiol. 1, 259. Isolation of floc-producing bacteria from activated sludge.

Meyer-Reil, L.A., 1981, Kieler Meeresforsch., Sonderh. 5, 311-317. Enzymatic decomposition of proteins and carbohydrates in marine sediments: methodology and field observations during spring.

Moore, P.G., Edwards, D.E. and Shirley, N.M. Standard Statistical Calculations, Pitman Publishing, London. 1972.

Morin, G., Fortin, J-P., Sochanska, W., Lardeau, J.P. and Charbonneau, R. 1979 Water Resources Research. 15, 1841-1850. Use of principal component analysis to identify homogeneous precipitation stations for optimal interpolation.

Morisawa, M.E., 1985. Geomorphology Texts, Rivers Form and Process Longman.

Munro, A.L.S., Williams, G.R. and Massie, L.C., 1973, Bull. ecol. Res. Commr. (Stockholm)., 17, 269-270. Seasonal variation in microbial activity.

Montgomery, H.A.C. and Hart, I.C. 1974. Water Pollution Control 73, 77-101. The design of sampling programmes for rivers and effluents.

Nannipieri, P., Ceccanti, B., Cervelli, S and Matarese, E. 1980. Soil Sci. Soc. Am. J., 44, 1011-1016. Extraction of Phosphatase, Urease, Protease, Organic Carbon and Nitrogen from Soil.

Nitkowski, M.F., Dudley, S. and Graikoski, J.T. 1977, Marine Pollut. Bull. 8, 276-279. Identification and characterisation of lipolytic and proteolytic bacteria isolated from marine sediments.

Norris, J.R., and Ribbons, D.W., (Eds) 1969-1976, Methods in Microbiology 1-9. Academic Press.

Nuttall, D., 1982a, J. Appl. Bact. 53, 49-59. The populations, characterisations and activity of suspended bacteria in the Welsh River Dee.

Nuttall, D., 1982b, J. Appl. Bact., 53, 61-71. The effects of environmental factors on the suspended bacteria in the Welsh River Dee.

Obst, U. 1985. Fresenius Z. Anal. Chem. 321, 166-168. Test instructions for measuring the microbial metabolic activity in water samples.

Oglesby, R.T., Carlson, C.A., and McCann, J.A. Eds. 1972, River Ecology and Man. Academic Press.

Painter, H.A., Denton, R.S. and Quarmby, C., 1968, Wat. Res. 2, 427-447. Removal of sugars by activated sludge.

Parsons, T.R. and Strickland, J.D.H., 1962. Deep-Sea Res. 8, 211-272. On the production of particulate organic carbon by heterotrophic processes in sea water.

Peterson, W.H., Fred, E.B. and Domogalla, B.P. 1925., Journal Biol. Chem. Vol. LXIII. The occurrence of amino acids and other organic nitrogen compounds in lake water.

Petit, N.M., Gregory, L.J., Freedman, R.B. and Burns, R.G. 1977. Biochimica and Biophysica Acta. 485, 357-366. Differential stabilities of soil enzymes, assay and properties of phosphatase and arylsulphatase.

Pettersson, K., 1980, Arch. Hydrobiol., 89, 54-87. Alkaline phosphatase activity and algal surplus phosphorus as phosphorus deficiency indicators in Lake Erken.

Pickaver, A.H. and Lyes, M.C., 1981, Estuarine, Coastal and Shelf Science, 12, 13-22. Aerobic microbial activity in surface sediments containing high or low concentrations of zinc taken from Dublin Bay, Ireland.

Pike, E.B., Carrington E.G. and Ashburner, P.A., 1972. J. Appl. Bact. 35, 309-321. An evaluation of procedures for enumerating bacteria in activated sludge.

Pike, E.B., in Ecological Aspects of Used-Water Treatment, 1975, Ed., Curds, C.R. and Hawkes, H.A. Volume 1. The Organisms and their Ecology. Academic Press.

Pirt, J. 1985. Catchment hydrology in Petts, G.E. and Foster, I.D.L. Eds. Rivers and Landscape. E. Arnold, London

Pomeroy, L.R., and Johannes, R.E., 1968, Deep-Sea. Res. 15, 381-391. Occurrence and respiration of ultraplankton in the upper 500 metres of the ocean.

Pomeroy, L.R. 1979, in Ecological Processes in coastal and Marine Systems. Ed. Livington, R.J. Plenum Press, New York. Secondary production mechanisms of continental shelf communities.

Prescott, J.M. and Willms, C.R., 1960, Proc. Soc. Exp. Biology and Med., 103, 410-413. Some characteristics of proteolytic system of a marine bacterial species.

Ratsep, A.I. and Sjogren, R.E. 1984, Freshwater Biology, 14, 423-429. Assay for biologically available phosphorus using proteolytic Aeromonas hydrophila

Rawlings, D.E. and Woods, D.R. 1978. J. Appl. Bact. 44, 131-139. Bacteriology and enzymology of fellmongery activated sludge systems.

Read, P.A., Renshaw, T., Jackes, A.D., Watson, P.G., and Anderson, K.J. 1977. Environ Pollut. 14, 275-287. Water quality in the Forth Estuary: a discriminant functional analysis.

Reichardt, W., Overbeck, J and Steubings, L., 1967. Nature, 216, 1345-1347. Free dissolved enzymes in lake waters.

Reichardt, W. 1979. Arch. Hydrobiol. Beih. Ergebn. Limnol. 12, 105-114. Significance of different methods to determine dehydrogenase (ETS) - activities in aquatic environments.

Richards, S.R., Eastwell, C. and Davies, M., 1984. Wat. Pollut. Control. 83, 300-313. the comparative estimation of 14 activated sludge plants using enzymatic techniques.

Riviere, J.W.M. la. 1972, in "Water Pollution Microbiology" ed. Mitchell. R., Wiley-Interscience, New York.

Robinson; M., 1986, J. Hydrology, 86, No. 1/2, 71-84. Changes in catchment runoff following drainage and afforestation.

Romanelli, A., et al 1980, Ann Sclavo. 22, 889. Detection and typing of non-tubercular Mycobacteria in urban waste wasters of Bergamo.

Ross, D.J. 1968. Pl. Soil. 28, 1-11. Some observations on the oxidation of glucose by enzymes in soil in the presence of toluene.

Ross, D.J. 1983. Soil Biol. Biochem. 15, 287-293. Invertase and amylase activities as influenced by clay minerals, soil-clay fractions and topsoils under grassland.

Ross, D.J., Tate, K.R., Cairns, A., Meyrick, K.F. and Pansier, E.A. 1982, New Zealand J. of Science 25, 341-349. Effects of slaughterhouse effluent and water on biochemical properties of two seasonally dry soils under pasture.

Sakurai, Y., 1967, J. Japan Biol. Soc. Water Waste. 2, 21-28. Investigation on the counting method of heterotrophic bacteria in the aquatic environment.

Satoh, Y., 1980, Hydrobiologia, 71, 233-237. Distribution of the urea decomposing bacteria in waters of Lake Suwa.

Savini, J. and Bodhaine, G.L., 1971, U.S. Geol. Survey Water Supply Paper. 1869, 59pp. Analysis of current meter data at Columbia River Gaging Stations, Washington and Oregon.

Sayler, G.S., Puziss, M. and Silver, M., 1979. Appl. and Environmental Microbiology, 38, 922-927. Alkaline phosphatase assay for freshwater sediments: application to pertubed sediment systems.

Shewan, J.M., Hobbs, G. and Hodgkiss, W., 1960, J. Appl. Bact., 23, 379-390. A determinative scheme for the identificjation of certain genera of gram-negative bacteria with special reference to Pseudomonadaceae.

Siddiqi, R.H., Engelbrecht, R.S. and Speece, R.E., 1966, Advances in Water Pollution Research. Proc 3rd int. conf. Wat. Poll. Res. Munich. 2, 353-380. The role of enzymes in the contact stabilisation process.

Simmons, J.S. 1926. Journal of Infectious Diseases, 39, 209. A culture medium for differentiating organisms of typhoid - colon aerogenes groups and for isolating certain fungi.

Singleton, F.L., et al 1982, Appl. Environ. Microbiol., 43, 1080-1084. Influence of salinity and organic nutrient concentration on survival and growth of Vibrio cholerae in aquatic microcosms.

Sizemore, R.K., Stevenson, L.H. and Hebeler, B.H. 1973. Distribution and activity of proteolytic bacteria in estuarine sediments. In Stevenson, L.H. and Colwell, R.R. Eds Estuarine Microbial Ecology. University of South Carolina Press, Columbia 1973.

Skujins, J. History of abiontic soil enzyme research in Burns, R.G. Ed. 1978. Soil Enzymes. Academic Press

Sokal, R.R. and Michener, C.D. 1958. Univ. Kansas. Sci. Bull, 38, 1409-1438. A statistical method for evaluating systematic relationships.

Sokal, R.R. and Sneath, P.H. A., 1963, Ed. Principles of Numerical Taxonomoy. W.H. Freeman and Co.

Somville, M. and Billen G., 1983, Limnol. Oceanogr., 28, 190-193. A method of determining exproteolytic activity in natural waters.

Somville, M. 1984, Applied and Environmental Microbiology. 48, 1181-1185. Measurement and study of substrate specificity of exoglucosidase activityy in eutrophic water.

Spier, T.W. and Ross, D.J. Soil Phosphatase and Sulphatase in Burns, R.G. Ed. Soil Enzymes 1978, Academic Press.

Spiro, R.G. 1966. Analysis of sugars found in glycoproteins. In. Colowick, S.P. and Kaplan, N.O. Eds. Methods of Enzymology. Vol. VIII 3-8 Academic Press. New York.

Sridhar, M.K.C. and Pillai, S.C., 1966, J. Sci. Industr. Res., 25, 167-171. Enzymes in sewage and sludges.

Sridhar, M.K.C. and Pillai, S.C. 1969, Eff. Wat. Treat. J. Feb. 1969 81-88. Catalase activity in polluted waters.

Sridhar, M.K.C. and Pillai, S.C., 1973. Water and Waste Treatment. August 1973. 35-42. Protease activity in sewage, sludges and effluents.

Sridhar, M.K.C. and Pillai, S.C., 1974, Wat. Waste. Treat. November 32 - 38. Urease activity in sewage, sludges and effluents.

Stavskaya, S.S. and Volchenkova, O.A., 1979, Water Resources, 6, 430, 437. Use of micro-organisms for treating polluted waters.

Stevens, R.J. and Parr, M.P., 1977, Freshwater Biology, 7, 351-355. The significance of alkaline phsophatase activity in Lough Neagh.

Straskrabova, V., 1974, International Revue des gesamten Hydrobiologie, 59, 9-11. Seasonal occurrence of several groups of heterotrophic bacteria in two reservoirs.

Streeter, H.W. and Phelps, E.B., 1925, Public Health Bulletin, 146. U.S. Public Health Service, Washington. A study of the pollution and natural purification of the Ohio River.

Suberkropp, K., and Klug, M.J., 1976, Ecology, 57, 707-719. Fungi and Bacteria associated with leaves during processing in a woodland stream.

Taga, N. and Kobori, H., 1978, Marine Biology, 49, 223-229. Phosphatase activity in eutrophic Tokyo Bay.

Takii, S., 1977, Wat. Res. 11, 85-89. Bacterial characteristics of activated sludges treating carbohydrate wastes.

Taylor-Eighmy, T. and Bishop, P.L., 1984, Applied and Environmental Micriobiology, 48, 1151-1158. Multiplicity of aspartate transport in thin wastewater biofilms.

Teuber M. and Brodisch, K.E.U., 1977, European J. Appl. Microbiol., 4, 185-194. Enzymatic activities of activated sludge.

Thiel, P.G. and Hattingh, W.H.J. 1967, Wat. Res. 1, 191-196. Determination of hydrolytic enzyme activities in anaerobic digesting sludge.

Toerien, D.F., 1967, Water Res., 1, 147-155. Enrichment culture studies on aerobic and facultative anaerobic bacteria found in anaerobic digesters.

Toerien, D.F., 1970. Water Res., 4, 129-148. Enzymatic patterns in two denitrifying microbial populations.

Toerien, D.F., Theil, P.G. and Hattingh, M.M., 1968, Water Research, 2, 505-513. Enumeration, isolation and identification of sulphate-reducing bacteria of anaerobic digestion.

Tyler, G., 1974, Plant and Soil, 41, 303-311. Heavy metal pollution and soil enzymatic activity.

Unz. R.F., 1971, Int. J. Syst. Bacteriol. 21, 91-99. Neotype strain of Zoogloea ramigera Itzigsohn.

Vaicum, L., Gruia, E. and Godeanu, S., 1965, Rev. Roum. Biochem., 2, 87-95. The determination of some enzymatic activities as a research method in activated sludge.

Vandeginste, B.G.M. and van Lersel, P.B.W., 1978, Proc. Analyt. Div. Chem. Soc., 15, 10-13. Study of the relations between biological, physical and chemical water quality description with pattern recognition techniques.

Verstraete, W., Voets, J.P. and van Lancker, P. 1976, Hydrobiologia, 49, 257-266. Evaluation of some enzymatic methods to measure the bio-activity of aquatic environments.

Verstraete, W. and Voets, J.P. 1977, Soil Biol. Biochem., 9, 253-258. Soil Microbial and Biochemical characteristics in relation to soil management and fertility.

Viessman, W., Knapp, J.W., Lewis, L.C. and Harbaugh, T.F. 1977. Introduction to Hydrology. Thomas Y Crowell Company Inc., New York.

Vives-Rego, J., Vaque, D. and Martinez, J., 1986, Wat. Res., 20, 1411-1415. Effect of heavy metals and surfactants on glucose metabolism, thymidine incorporation and exoproteolytic activity in sea water.

Vollenweider, R.A. (Ed), 1974, I.B.P. Handbook No 12, 2nd Edition, Blackwell Scientific Publications. A manual on methods for measuring primary production in aquatic environments.

Wainwright, M. and Duddridge, J.E. 1982, European J. Appl. Biotechnol. 15, 241-245. Effects of heavy metals on enzyme synthesis in substrate - amended river sediments.

Waksman, S.A., Carey, C.L. and Renszer, W.H. 1933. Biol. Bull. (Woods Hole), 65, 57-79. Marine bacteria and their role in the cycle of life in the sea. Decomposition of plant and animal residues by bacteria.

Ware, G.C. and Mellon, M.A., 1956, J. Hyg., Camb., 54, 99-101. Some observations on the coli/coli-phage relationships in sewage.

Warn, A.E. and Brew, J.S., 1980, Water Research, 14, 1427-1439. Mass Balance.

Webster, R.M., 1981, Process Biochemistry October/November 1981, 12, Microbial ecology of river deposits in the Rivers Kennet and Thames at Reading, England.

Weeks, D.B., 1969. J. applied Bact. 32, 13-18. Problems concerning the relationships of Cytophagas and Flavobacteria.

Wetzel, R.G., 1975, Limnology, Saunders.

Whipple, G.C., Fair, G.M., and Whipple, M.C., 1927, The Microscopy of Drinking Water, J. Wiley and Sons.

Williams, P.J. le B., 1973, Limnol. Oceanogr., 18, 159. The validity of the application of simple kinetic analysis to heterogeneous microbial populations.

Winberg, G.G. (ed), 1971, Symbols, Units and Conversion Factors in Studies of Fresh Water Productivity. London: International Biological Programme.

Winkler, M.A. Application of the principles of fermentation engineering to biotechnology in Principles of Biotechnology Ed. Wiseman, A. Surrey University Press, 1983.

Witthauer, D.P., 1980, European J. Appl. Microbiol. Biotechnol., 9, 151-16 . Biocoenosis and degradation in model wastewater treatment plants.

Wolman, M.G., 1967, Geogr. Ann. Ser. A., 49, 385-395. A cycle of sedimentation and erosion in urban river channels.

Wooldridge, W.R. and Standfast, A.F.B. 1936. J. Biochem. 30, 1542-1553. The role of enzymes in activated sludge and sewage oxidations.

Wright, R.T. and Hobbie, J.E., 1965, Limnol. Oceanogr., 10, 22-28. The uptake of organic solutes in lake water.

Wuhrmann, K., 1964, River bacteriology and the role of bacteria in self-purification. In Principles and Applications in Aquatic Microbiology eds Heukelekian, H. and Dondero, N.C. John Wiley and Sons.

Yoshikura, T., et al 1981, Bull, Jpn. Soc. Sci. Fish., 47, 769-774. Relationships between bacterial flora and organic pollution in river waters.

Zanella, E., Conkey, J. and Tesmer, M., 1978, Tappi, 61, 61-65. Non-settleable solids. Characteristics and use by two aquatic food chain organisms.

Zobell, C.E. and Feltham., 1938, J. Mar. Res. 4, 312-327. Bacteria as food for marine invertebrates.

Zobell, C.E. and Feltham, 1942, Ecology, 23, 69. The bacterial flora of a marine mud flat as an ecological factor.

Zobell, C.E. and Grant, C.W., 1943, J. Bact., 45, 555. Bacterial utilisation of low concentrations of organic matter.

Zobell, C.E., 1943, J. Bact., 46, 39-56. The effect of solid surfaces upon bacterial activity.

Iturriaga, R., 1979, Marine Biology, 55, 157-169. Bacterial activity related to sedimenting particulate matter.

Itzigsohn, H. 1868, S.B. Gesellschaft naturf Freunde, Berlin. Entwicklungsvorgange von Zoogloea. Oscillaria. Synedra. Staurastrum, Spirotaenia and Chroolepus.

APPENDIX 1

Sampling Dates for River Chelt Sites

DATE	SANDFORD MILL	WITHY BRIDGE	HAYDEN EFFLUENT	BODDINGTON BRIDGE	INCHMOOR BRIDGE
29.04.81		x		x	
06.05.81		x		x	x
14.05.81		x		x	x
15.07.81		x		x	x
19.08.81	x	x		x	x
27.08.81	x	x		x	x
28.10.81	x	x		x	x
18.11.81	x	x		x	x
02.12.81	x	x		x	x
16.12.81	x	x		x	x
22.01.82	x	x	x	x	x
05.02.82	x	x	x	x	x
15.02.82			x	x	x
05.03.82		x	x	x	
26.3.82		x	x	x	x
06.04.82				x	
07.05.82				x	
14.05.82		x	x	x	x
26.05.82	x	x	x	x	x
09.06.82				x	
18.06.82		x	x	x	
02.07.82	x	x	x	x	x
16.07.82	x	x	x	x	x
06.08.82	x	x	x	x	
04.06.83				x	
24.06.83		x	x		
01.07.83				x	
08.07.83		x			
25.05.84				x	

APPENDIX 1 continued

Sampling Dates for River Chelt Sites

DATE	SANDFORD MILL	WITHY BRIDGE	HAYDEN EFFLUENT	BODDINGTON BRIDGE	INCHMOOR BRIDGE
22.06.84				x	
11.01.85				x	
19.01.85		x			
08.05.85		x	x	x	
30.05.85				x	
13.06.85			x	x	
21.06.85				x	
02.08.85				x	

APPENDIX 2

Summary Data on Major Sites
Mean Value and Range (Sample Numbers)

DETERMINAND	R CHELT AT WITHY BRIDGE	HAYDEN EFFLUENT	R CHELT AT BODDINGTON BRIDGE
Temperature ^{o}C	10.7 (25) 2.5 - 18.0	14.4 (13) 8.5 - 19.5	12.8 (34) 5.0 - 20.0
Discharge Ml/d	19.8 (28) 2 - 97	36.7 (14) 27 - 57	57 (34) 30 - 152
Saccharase Unit See Below	1202 (22) 3 - 920	2599 (13) 1394 - 3686	1496 (32) 476 - 4106
Protease Units See Below	770 (21) 94 - 1231	5156 (13) 2360 - 8604	3613 (33) 1304 - 8295
BOD (ATU) mg/l	2.5 (21) 1.1 - 8.1	16.6 (12) 12 - 20	8.5 (27) 3.0 - 18.8
Suspended Solids mg/l	17.2 (23) 2 - 180	21 (12) 11 - 32	23.2 (29) 5.0 - 150
Ammonia as N mg/l	0.218 (19) 0.05 - 1.7	6.2 (9) 4.3 - 8.0	3.7 (23) 0.6 - 6.6
Oxidised Nitrogen as N mg/l	5.3 (19) 3.4 - 6.5	17.7 (9) 11.6 - 22.4	12.8 (23) 8.8 - 17.8
pH	8.1 (15) 7.5 - 8.6	7.7 (6) 7.5 - 7.9	7.9 (18) 7.6 - 8.4
Plate Count CFU/ml	$1.9x10^{5}$ (20) $2.7x10^{4}$-$6.8x10^{5}$	$5.0x10^{6}$ (11) $1.4x10^{5}$-$1.1x10^{7}$	$1.7x10^{6}$ (27) $9.1x10^{4}$-$9.3x10^{6}$

Saccharase units ng reducing sugar released/ml sample/hour.
Protease units ng tyrosine released/ml sample/hour.